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The role of oxidation-sensitive nociception and epigenetics in endometriosis-associated pain

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THE ROLE OF OXIDATION-SENSITIVE NOCICEPTION AND EPIGENETICS IN ENDOMETRIOSIS-ASSOCIATED PAIN

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APPROVAL OF DISSERTATION

We, the faculty supervising the work of Kristeena Ray Wright, affirm that the dissertation, *The role of oxidation-sensitive nociception and epigenetics in endometriosis-associated pain*, meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences Graduate Program. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

I dedicate this thesis to Michael and Liana, my anchor and rock. Thank you for being there to share in the good, the bad, and the downright nerdy. Baby girl, I hope that one day you understand what a critical role you played in the completion of this work. I love you both to the moon and back.

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ABSTRACT

Endometriosis is an enigmatic disease that is associated with severe symptoms and consequences such as total hysterectomy. Upwards of 10% of women of reproductive age have endometriosis, most of whom are afflicted with chronic pelvic pain and/or infertility. Despite many treatment options available to endometriosis patients, there is no cure for this condition and recurrence of symptoms is extremely common. In CHAPTER 1, we describe the mechanisms involved in endometriotic pain and the currently available treatment strategies. Our laboratory has provided evidence for the role of oxidative stress in the etiology of endometriosis and its associated pain. In this dissertation research, we wanted to specifically understand the mechanistic role for the abundance of oxidized lipoproteins (ox-LDLs) in the peritoneal fluid (PF) of women with endometriosis. We hypothesize that the ox-LDLs and the peritoneal milieu play a dynamic role in endometriosis. These components are epigenetic modulators of inflammatory and nociceptive processes. Our IRB-approved study used eutopic and ectopic endometrial tissues and peritoneal fluid from patients with (endo) and without (control) endometriosis (IRB-[114954-20](9074)). Tissue sources included eutopic endometrium of control patients (EuNN), eutopic endometrium from patients with endometriosis (EuYY), and ectopic tissue from women with endometriosis (EcYY). Peritoneal fluid (PF), was collected from patients categorized as having neither endometriosis nor pain (NN-PF), endometriosis and pain (YY-PF), or endometriosis without pain (YN-PF). In CHAPTER 2, we provide evidence for the ability of the ox-LDL components of PF to induce inflammation and nociception in Sprague-Dawley rats (IACUC 485[397498-7]). The ox-LDL nociceptive and inflammatory responses were similar to that observed with PF from women with endometriosis and pain. Antioxidants were able to alleviate these nociceptive responses. In CHAPTER 3, we provide a mechanism by which these ox-LDL components

induce inflammatory and nociceptive responses. Global miRNA expression was measured in tissues and PF-treated cells and key miRNAs and their targets were validated by qPCR. Several miRNAs (e.g. isoforms of let-7, miR-98, and miR-374) and their gene targets (e.g. IL-10, Mip1α, and MCP1) were differentially expressed in both ox-LDL and endo PF-treated cells. In CHAPTER 4, an epigenetic mechanism involving Enhancer of zeste 2 (EZH2) is proposed to contribute to endometriosis and associated pain. RT-qPCR and Western blots were used to measure the expression of key epigenetic factors in patient tissues and PF-treated cells while ChIP-qPCR identified interactions between upstream regulator Jumonji protein 2 and epigenetic genes. This study provides mechanistic evidence for oxidized lipoprotein components playing a role in endometriosis associated pain. We also provide evidence for epigenetic changes in the endometriosis pain. Future studies will test drugs that target oxidation and/or epigenetic pathways in animal models of endometriosis and patients with endometriosis.

CHAPTER 1

ENDOMETRIOSIS-ASSOCIATED PAIN

This manuscript is a revised version of *Power over pain: A brief review of current and novel interventions for endometriosis-associated pain*

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ABSTRACT

An estimated 10%-15% of women of reproductive age suffer from endometriosis and can be plagued with one or many forms of pain. It is no mystery that endometriosis is an extremely complex disease, with several factors leading to the predominant symptoms of infertility and pain. Although there are currently multiple options available for treating endometriosis-associated pain, none can completely relieve the symptoms. This review both highlights the current trends in treatment of endometriosis-associated pain and explores some novel options available for therapy directed towards oxidative stress, inflammation and nociceptive mechanisms of pain. A PubMed search was conducted to identify the most recent publications on the topic of pain associated with endometriosis, and further research was performed to clarify the

mechanism by which current treatments target pain. Lastly, the authors include a review of pharmacological options at the forefront of endometriosis research. A more comprehensive understanding of the mechanisms behind endometriosis-associated pain will ultimately lead to more effective treatments and improved prognoses for patients.

INTRODUCTION

Endometriosis is defined by the presence of endometrial tissue in ectopic locations, typically in or around the peritoneal cavity (Burney & Giudice, 2012; Ciarmela, Critchley, Christman, & Reis, 2013; Vercellini, Vigano, Somigliana, & Fedele, 2014). While the exact prevalence of endometriosis is likely underrepresented, most sources cite a minimum of 10% of women in their reproductive years (Ciarmela et al., 2013; Platteeuw & D'Hooghe, 2014; Young, Brown, Saunders, & Horne, 2013). While 20-25% of endometriosis patients are asymptomatic (Bulletti, Coccia, Battistoni, & Borini, 2010), others experience a number of debilitating symptoms. The most predominant symptoms are infertility and pelvic pain, the latter being considered the "classic symptom" of the condition and the primary reason for medical visits by patients with endometriosis (Fraser, 2010). In 1985, the American Fertility Society (AFS) made its latest revisions to the classification system for endometriosis ("Revised American Fertility Society classification of endometriosis: 1985," 1985), followed by the ASRM classifications proposed in 1996 ("Revised American Society for Reproductive Medicine classification of endometriosis: 1996," 1997). At this time, these committees were aware of the need to incorporate pain into their classification systems and recognized that attempts were being made to link pain to the location and/or severity of endometriosis ("Revised American Society for Reproductive Medicine classification of endometriosis: 1996," 1997). However, it is likely that at that time, the association between pain and endometriosis had not been fully elucidated. At the

time of the AFS revisions, neither pain nor the etiology of pain associated with endometriosis was taken into account (Roberts & Rock, 2003). Hence this lack of knowledge is now problematic, considering the inability to determine the etiology of this associated pain is the primary roadblock in designing and providing effective treatment for patients with endometriosis. This review will provide a brief overview of the current treatment options available for endometriosis-associated pain (the readers are encouraged to read recent in depth reviews (Brown & Farquhar, 2014; Kobayashi et al., 2014; Morotti, Vincent, Brawn, Zondervan, & Becker, 2014; Platteeuw & D'Hooghe, 2014; Practice Committee of the American Society for Reproductive, 2014; Stratton & Berkley, 2011) on this topic). The major emphasis of this review is to address some of the new and novel pain mechanisms involved in the etiology of pain in endometriosis, which may help in developing better treatment options for this disease.

CURRENT TREATMENTS AND THEIR EFFECTIVENESS IN TREATING PAIN

Hormonal Therapies

Endometriosis has long been considered a hormonal disorder, with high levels of estrogen contributing to the development and growth of endometriotic lesions (Arici, Tazuke, Attar, Kliman, & Olive, 1996; Giudice & Kao, 2004; Kitawaki et al., 2002). At the molecular level, endometriosis-related inflammation is attributed to excessive estrogen production, as it promotes the secretion of several inflammatory cytokines and growth factors as well as prostaglandin E_2, or PGE_2 (the cyclooxygenase product and a major pain inducing molecule) (Ferrero et al., 2014). Oral contraceptives (OCs), commonly referred to as "the pill," work to block ovulation in conjunction with deprivation of estrogen to the endometrium and endometriomas (Maia, Haddad, & Casoy, 2013). This mechanism relieves pain for as long as the active pills are being consumed, with the pain often returning upon cessation (Practice

Committee of American Society for Reproductive, 2008). Side effects are relatively minor, but OCs do, of course, prevent conception.

Progestins and progesterone can be administered orally, by intramuscular depot injection, or via an intrauterine device (Schweppe, 2012). It has been proposed that the mechanism of action of progestins in relation to endometriosis involves reducing metalloproteinases and growth factors (Schweppe, 2012) as well as inducing atrophy of the endometrial tissue. Lesser discussed mechanisms associated with progestins and progestogens are the suppression of inflammatory reactions and estrogen in and around endometriomas (Schweppe, 2012). The introduction of dienogest has allowed for further research into progestogens as the first-line of treatments for endometriosis and its associated pain (Petraglia et al., 2012; Strowitzki, Marr, Gerlinger, Faustmann, & Seitz, 2012). While use of progestins are effective means of relieving endometriosis-related pain, the drawback is that they also prevent pregnancy and any breakthrough bleeding is often accompanied by pain.

GnRH Analogs and Danazol

GnRH agonists such as leuprolide acetate are more effective at inducing amenorrhea than the afore-mentioned treatments and also promote decidualization and atrophy of the endometrial tissue (Practice Committee of the American Society for Reproductive, 2014). For this reason, a growing number of studies are being performed to investigate new GnRH agonist options. A recent study by Leone Roberti Maggiore and colleagues detailed the role of triptorelin in treating endometriosis-associated pain (Leone Roberti Maggiore et al., 2014)**.** As with all GnRH agonists, triptorelin depresses this pain by depriving the lesions of the estrogen that allows them to flourish (Gargiulo & Hornstein, 1997; Leone Roberti Maggiore et al., 2014). Unfortunately, the hypoestrogenic environment caused by these treatments leads to a menopause-like state in

the patients (Leone Roberti Maggiore et al., 2014; Practice Committee of American Society for Reproductive, 2008). To combat this, the concept of adding estrogen/progestogen to this treatment has now become the gold standard ("Practice bulletin no. 114: management of endometriosis," 2010; Practice Committee of the American Society for Reproductive, 2014; Zupi et al., 2004).

Danazol is an androgen that prevents the release of steroidal hormones. By lowering the levels of estrogen available to endometriotic lesions, danazol leads to their atrophy and lowers endometriosis-associated pain (Barbieri, 1990; Selak, Farquhar, Prentice, & Singla, 2007). However, because this drug is an androgen, it also causes undesirable side effects such as hirsutism, acne, and weight gain. In terms of endometriosis pain relief, danazol is as effective as GnRH analogs (Selak et al., 2007).

Aromatase Inhibitors

Aromatase is the key enzyme in the synthesis of estrogens from androgens. Aromatase inhibitors, while not approved by the US Food and Drug Administration for endometriosis, are thought to alleviate endometriosis-associated pain with their ability to lower recurrence rates or postpone recurrence more effectively than other first-line alternatives (Practice Committee of the American Society for Reproductive, 2014). However, to combat some adverse effects of these drugs, adjuvant therapies such as progestogens and OCs have been shown to improve pain in women of reproductive age (Ailawadi, Jobanputra, Kataria, Gurates, & Bulun, 2004; Amsterdam et al., 2005; Pavone & Bulun, 2012). In post-menopausal endometriosis patients, there is evidence that an aromatase inhibitor alone may be the better option for pain treatment (Pavone & Bulun, 2012).

Analgesics

Non-steroidal anti-inflammatory drugs (NSAIDS) block the action of cyclooxygenase (COX-2), the enzyme that produces prostaglandin E2. Both PGE_2 and COX-2 are present at higher levels both in the endometriotic tissue and the PF of patients with endometriosis than in healthy women (Badawy, Marshall, & Cuenca, 1985; Cobellis et al., 2004; M. H. Wu, Lu, Chuang, & Tsai, 2010). These agents are the most commonly prescribed drugs to relieve endometriosis-associated dysmenorrhea and other pain symptoms (Nasir & Bope, 2004; "Practice bulletin no. 114: management of endometriosis," 2010). However, new reports indicate that several of these pain symptoms may not be significantly reduced by these drugs (Barcena de Arellano & Mechsner, 2014; Practice Committee of the American Society for Reproductive, 2014) .

Surgical Procedures

Laparoscopic surgery is routinely performed as a diagnostic and a treatment option for endometriosis (Duffy et al., 2014; Practice Committee of the American Society for Reproductive, 2014; Vercellini et al., 2014). During the surgery, typically the peritoneal fluid is drained from the peritoneal cavity, as it is a dynamic milieu of inflammatory and nociceptive molecules that exacerbate the symptoms of endometriosis and pain (Koninckx, Kennedy, & Barlow, 1998; Morotti et al., 2014; Santanam, Murphy, & Parthasarathy, 2002). In the incidence that endometriotic lesions are identified within the peritoneal cavity, they are excised at the time of laparoscopy. While laparoscopy is shown to relieve endometriosis-associated pain, it is common for the pain to return within a few months or years after the procedure (Giudice, 2010). This recurrence of aggravating symptoms often leads to the removal of reproductive organs on which endometriotic lesions may develop and flourish. A hysterectomy entails removing the

uterus while *total hysterectomy* typically entails removal of the uterus, fallopian tubes, and ovaries ("Practice bulletin no. 114: management of endometriosis," 2010). While hysterectomy does not guarantee relief from all pain, it has proven to be very successful and is the ultimate treatment in 20 to 30% of endometriosis cases (Acien & Velasco, 2013; Shah & Adlakha, 2014). Other surgical procedures such as neurectomy and laparoscopic uterine nerve ablation have been developed to address the fact that pain is a neurological phenomenon. The readers are referred to the comprehensive review by Stratton and colleagues on how endometriosis affects the central nervous system and current methods (both surgical and pharmacological) for treating the resultant pain (Stratton & Berkley, 2011).

Roadblocks to Treatment

Though several investigators continue to search for better treatments for this enigmatic disease, there still remain roadblocks for success. Without intervention, endometriosis can last the lifetime of a woman, from her teenage years until her 70s. It affects both her health and quality of life. Effective treatment of endometriosis and its associated symptoms is therefore a key to improving her quality of life. Despite the several available treatment options, combating the pain associated with this condition and the disease itself remains unsuccessful. The recurrence of pelvic pain following the afore-mentioned treatments is the primary reason for continued investigations into identifying better therapeutic options for patients with endometriosis. Because many of the women suffering from this condition are young, hysterectomies are typically undesirable and treated as a last resort. There is now an urgent need to identify and define the underlying causes of endometriosis and its associated symptoms such as the debilitating pain associated with it. In 2009, the recommendations of an international consensus group on endometriosis research were published (Rogers et al., 2009). Amongst other

goals, they stated that the "development of non-hormonal medical treatments to prevent or treat endometriosis and associated symptoms is a priority (Rogers et al., 2009)." In this review, we highlight some of new mechanisms that may be involved in pain associated with endometriosis.

NOVEL MECHANISMS OF PAIN

At this point, we know that at least five types of endometriosis-associated pain have been identified: (i) menstrual cycle pain, (ii) perimenstrual pain (dysmenorrhea), (iii) nerve entrapment, (iv) neuropathic pain, and (v) other pain due to hyperalgesia and allodynia (Fraser, 2010). Our laboratory has been working to uncover some of the nociceptive mechanisms that cause hyperalgesia and allodynia. While the categories of pain associated with endometriosis are well-defined, the mechanics behind each is rather complex. The Practice Committee of the American Society for Reproductive Medicine (ASRM) recently reported that the three primary mechanisms of endometriosis-associated pain are: (i) the production of cytokines and growth factors from the cells of the implants (i.e. macrophages), (ii) the effects of active bleeding from these implants, and (iii) irritation of invasion of the pelvic floor nerves by the implants (Practice Committee of the American Society for Reproductive, 2014). Here we propose some additional mechanisms of pain as well as potential therapies for pain experienced by women with endometriosis.

Oxidative Stress

There is an abundance of evidence supporting the role of oxidative stress in the development and progression of endometriosis, including studies from our laboratory (Murphy, Santanam, & Parthasarathy, 1998; Santanam, Kavtaradze, Murphy, Dominguez, & Parthasarathy, 2013; Santanam, Murphy, et al., 2002). As in other painful disorders such as myofascial pain disorder (Koca et al., 2014) and fibromyalgia (Fatima, Das, & Mahdi, 2013),

endometriosis has been associated with an increase in reactive oxygen species (ROS) produced by macrophages (Lousse et al., 2012; Murphy, Santanam, & Parthasarathy, 1998; Santanam, Murphy, et al., 2002). There is an abundance of oxidation sensitive markers in the PF of women with endometriosis (Carvalho, Samadder, Agarwal, Fernandes, & Abrao, 2012; Santanam, Murphy, et al., 2002). We have shown that, in patients with endometriosis, there are higher levels of oxidized lipids such as lysophosphatidylcholine (Murphy, Santanam, & Parthasarathy, 1998) and oxidized lipoproteins, especially oxidized low-density lipoprotein (ox-LDLs) (Murphy, Santanam, & Parthasarathy, 1998; Santanam, Murphy, et al., 2002). These oxidized lipoproteins increase the proliferation of endometrial cells and increase recruitment of macrophages in the peritoneal cavity (Murphy, Santanam, & Parthasarathy, 1998; Santanam, Murphy, et al., 2002). Components of oxidized low density lipoprotein such as hydroperoxy eicosatetraenoic acids (HPETES) or hydroperoxy octadecaenoic acids (HPODEs) express prostaglandin-like properties (Proudfoot, Beilin, & Croft, 1995) and hence have the ability to participate in nociceptive pathways (Patwardhan et al., 2010; Patwardhan, Scotland, Akopian, & Hargreaves, 2009). We recently demonstrated that components of oxidatively-modified LDLs were similar to the oxidized lipids (including prostaglandin products) present in the peritoneal fluid of women with endometriosis. These oxidized components of LDL were capable of causing hyperalgesia in rodent models (K. Ray et al., 2015).

In addition to their direct nociceptive actions, increased ox-LDLs also induce pro-inflammatory cytokines in the peritoneal fluid (PF). Cytokines such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and macrophage colony-stimulating factor (M-CSF) are present at increased concentrations in the PF of patients with endometriosis (Polak, Barczynski, et al., 2013). Ox-LDL also increased monocyte chemotactic protein 1 (MCP-1) in endometrial cells

(Rong, Ramachandran, Santanam, Murphy, & Parthasarathy, 2002). The abundant presence of these molecules in the PF of women with endometriosis and their ability to (i) increase proliferation of endometrial cells (ii) participate in nociceptive pathways and (iii) increase macrophage mediated inflammation, makes them an ideal candidate for targeting in endometriosis associated pain. Many studies have brought oxidative stress to the forefront of endometriosis research and Table 1.1 summarizes their key findings.

Inflammatory/Immune Response

The afore-mentioned cytokines (TNF-α, IL-6, M-CSF) have also been linked to the tissues of endometriosis patients. It is well established that much of the inflammatory response seen in endometriosis is linked to the role of nuclear factor-kappa B (NF-κB), cyclooxygenase 2 (COX-2), and aromatase within the eutopic endometrium (Brenner, Nayak, Slayden, Critchley, & Kelly, 2002; Maia et al., 2013; Ponce et al., 2009). The cytokines generated through the activation of these pathways participate in the nociceptive responses (Garmendia & De Sanctis, 2012). However, these do not directly contribute to endometriosis-associated nociception. A recent animal study by Alvarez and Levine led to the implication of IL-6 and neural growth factor (NGF) in this pain, but not TNF-α. The authors of this study proposed that while IL-6 acts to sensitize nociceptors at their receptors, it is the NGF which is released from endometriotic lesions that contributes to mechanical hyperalgesia (Alvarez & Levine, 2014).

Based on the definition of endometriosis, it is reasonable to think that endometriotic implants may be viewed by the immune system as a foreign entity. In fact, activated monocytes or macrophages are seen in higher numbers in the PF of women with endometriosis than in those without (Agic et al., 2006; Bedaiwy & Falcone, 2003; Santanam, Murphy, et al., 2002). Macrophages are also at least partially responsible for the development and growth of

endometriotic lesions (Capobianco & Rovere-Querini, 2013; Santanam, Murphy, et al., 2002). While our studies have shown that the scavenging function of these activated monocytes is thought to be compromised in an endometriotic environment (J. G. Kim, Keshava, Murphy, Pitas, & Parthasarathy, 1997; Santanam, Murphy, et al., 2002), these immune cells continue to secrete cytokines and growth factors which links these macrophages to neuropathic pain. This link is a major area of investigation in several clinical conditions including endometriosis (Baron, Binder, & Wasner, 2010). A combination of inflammatory and neuropathic processes is present in patients with endometriosis, creating what Barcena de Arellano and colleagues termed "a neurogenic inflammatory reaction." As peritoneal endometriotic lesions release an array of pain mediators, macrophages and mast cells release a number of cytokines and chemokines and critical peripheral nerve fibers release pro-inflammatory neurotransmitters that potentiate pain (Barcena de Arellano & Mechsner, 2014; Kobayashi et al., 2014).

Nociceptive Pain

Nociceptive pain differs from neuropathic pain in that it results from neural activity that is secondary to the tissue damage itself. Typically pain generated in endometriosis is categorized as neuropathic or inflammatory; however, researchers often note a change in nociceptive pain when discussing the effects of a potential treatment. Simsek et al stated that atorvastatin lowers hypernociception in endometriosis animal models (Simsek et al., 2014). The nociceptive mechanisms in such cases have yet to be elucidated, but there are other molecules that have been shown to be involved in endometriosis. The role of fractalkine (CX3CL1) and its receptor (CX3CR1) in endometriosis has been the topic of multiple studies (Bellelis et al., 2013; Shimoya et al., 2005; Y. Wang et al., 2013; Y. Wang et al., 2014). While there seems to be conflicting reports of its role in the peritoneal fluid (Shimoya et al., 2005)Wang and colleagues have

established the role of fractalkine in the cross-talk between endometrial stromal cells (ESCs) and macrophages in endometriosis patients (Y. Wang et al., 2014). Eutopic ESCs in these patients secrete higher levels of fractalkine that contribute to increased invasiveness of the cells. The inflammatory chemokine interleukin-22 (IL-22) of the IL-10 family, along with its receptors, is also overexpressed in eutopic endometrium and ectopic lesions of endometriosis patients compared to healthy patients (Y. Guo et al., 2013). The same study also concluded that IL-22 is responsible for promoting ESC proliferation (Y. Guo et al., 2013). These inflammatory chemokines and cytokines have a role in nociception and are now considered as potential targets for treating pain (Fedele & Berlanda, 2004; Kyama et al., 2009; Neziri et al., 2014; Schwager et al., 2011). Transient receptor potential vanilloid type 1, or TRPV1, also acts as a nociceptive mediator in endometriosis-associated pain (Greaves, Grieve, Horne, & Saunders, 2014; Rocha et al., 2011; Song, Leng, & Lang, 2012). Higher TRPV1 immunoreactivity was discovered in peritoneal endometriomas from patients with chronic pelvic pain compared to those without chronic pelvic pain (Rocha et al., 2011). Significantly higher TRPV1 expression has also been directly linked to increased pain scores in endometriosis patients (Song et al., 2012). Lastly, endogenous opioids have long been associated with nociceptive pain (Basbaum & Fields, 1984; Millan, 2002). Matsuzaki and colleagues found that the expression of the mu-opioid receptor (MOR) is higher in ectopic endometrium than the eutopic counterpart in both ovarian and deep infiltrating endometriosis (Matsuzaki et al., 2006). While the function of the MOR in the disease is unclear, it has been shown that GnRH agonists as well as progestin therapies lowered MOR expression in endometriosis (Matsuzaki et al., 2007). Agents targeting the afore-mentioned genes could potentially be considered as treatments for endometriosis; however, the potential for habit-forming side effects for some of these agents may be a major downside to these options.

MicroRNAs and Other Epigenetic Mediators

Epigenetics describes heritable genetic alterations that do not involve any changes to the DNA sequence (Holliday, 1994). This area of research is on the rise in many disorders, including endometriosis. At the forefront of epigenetic research is the role of microRNAs (miRNAs), short RNAs (about 23 nucleotides) which are capable of regulating gene expression at the transcriptional, post-transcriptional, and translational levels by binding to complementary sequences on target mRNA (Andersen, Duroux, & Gazerani, 2014; Bartel, 2009). More specifically, miRNA regulation occurs via degradation of the target mRNA in cases of perfect complementarity with the target mRNA sequence, or via impaired translation in cases of imperfect matching, leading to gene silencing (Deng, Calin, Croce, Coukos, & Zhang, 2008; Mari-Alexandre et al., 2016). MiRNAs are also capable of activating gene expressions via targeting the mRNA or by repressing nonsense-mediated RNA decay (Vasudevan, Tong, & Steitz, 2007). MiRNAs are being studied by many as biomarkers in endometriosis (Braza-Boils et al., 2014; Ohlsson Teague et al., 2009; Teague, Print, & Hull, 2010), but they could also be key biomarkers in endometriosis-associated pain. The downregulation of miRNAs associated with inflammation could cause heightened expression of key inflammatory genes. Laudanski and colleagues found significant downregulation of miR-483-5p, which targets IGF2, and miR-629-3p, which is linked to inflammation, in the eutopic endometrium of endo patients compared to control tissues (Laudanski et al., 2013; Mari-Alexandre et al., 2016). Further studies could determine whether these miRNAs could be the targets of therapies for chronic pelvic pain associated with endometriosis. Circulating miRNAs could also be targeted for therapeutic purposes. As in certain cancers, these miRNAs can bind toll-like receptors (TLRs) of immune cells.

Histone modifications and DNA methylation are other aspects of epigenetics and offer additional mechanisms by which pain may afflict endometriosis patients. Methylation of a gene's promoter region is typically associated with repression of its expression. There is a possibility that proinflammatory cytokines trigger aberrations in the epigenetic and genetic profile of endometriosis patients. For example, TNFα is at least partially responsible for the hypermethylation of the progesterone receptor isoform B (PR-B) promoter (S. W. Guo, 2009; Y. Wu, Strawn, Basir, Halverson, & Guo, 2006). Limiting the number of progesterone receptors could further contribute to the progression of endometriosis and render some hormonal therapies useless.

POTENTIAL NOVEL TREATMENTS

Antioxidants

Due to the implication of oxidative stress in several forms of chronic pain, numerous researchers have sought to treat painful disorders with antioxidants. Vitamins E and C supplementation for 4 to 8 weeks before laparoscopic surgery relieved dysmenorrhea and dyspareunia symptoms in women with endometriosis (Santanam et al., 2013). After as little as two months of Vitamin E and C administration also lowered inflammatory markers [regulated on activation, normal T cell expressed and secreted (RANTES), interleukin 6 (IL-6), monocyte chemotactic protein (MCP-1)] in the PF of patients with endometriosis (Santanam et al., 2013). In the rodent model of nociception, we observed that antioxidants (vitamin E or n-acetylcysteine) inhibited the oxidized-LDL mediated nociceptive responses (unpublished observations). Similarly, antioxidants such as melatonin or n-acetyl cysteine showed promises of reducing endometriosis-associated pain in human subjects (Porpora et al., 2013; Schwertner et al., 2013). At low doses antioxidants can serve as a safe, alternative medical treatment (Porpora et al., 2013;

Santanam et al., 2013; Schwertner et al., 2013). Recently, several of the Chinese herbs that have antioxidant properties are also being considered as adjunct treatment for endometriosis (Flower, Lewith, & Little, 2011; Jiang, Shen, & Wang, 2010). These studies suggest a likelihood of using antioxidant treatments as a stand-alone or adjuvant therapy to the currently available treatments for endometriosis.

Alternate Anti-inflammatory Agents

Currently non-steroidal anti-inflammatory drugs (NSAIDs) are the most common anti-inflammatory treatment for endometriosis (Nasir & Bope, 2004; "Practice bulletin no. 114: management of endometriosis," 2010). However, as mentioned earlier, confidence in the effectiveness of these drugs to treat endometriosis-associated dysmenorrhea is waning (Allen, Hopewell, Prentice, & Gregory, 2009; Brown & Farquhar, 2014; Practice Committee of the American Society for Reproductive, 2014). Other new agents have been identified which have the potential for more effectively alleviating these symptoms. One such agent is Parthenolide, the bioactive component of feverfew, which has been shown to suppress the progression of endometriosis by targeting the NF-κB inflammatory pathway. Takai et al. found that this compound diminished TNF-α-induced IL-8 gene and protein expression, while also down-regulating COX-2 gene expression and PGE_2 synthesis in human ESCs (Takai et al., 2013). At this point, this feverfew derived compound is not associated with any severe side effects. A phase I trial is currently underway. Another natural anti-inflammatory agent, derived from the bark of the French maritime pine, blocks the activity of both COX-1 and COX-2 to produce anti-inflammatory and anti-thrombotic effects (Kohama, Herai, & Inoue, 2007; Maia et al., 2013). This compound, eventually dubbed Pycnogenol®, also down-regulates NF-κB gene expression. These properties prompted Kohama and colleagues to investigate the compound's effectiveness

as a treatment for menstrual pain (Kohama, Suzuki, Ohno, & Inoue, 2004). After unveiling its potential as an analgesic, a clinical trial was conducted to test the efficacy of Pycnogenol® as a treatment for endometriosis. When compared to a GnRH agonist, Pycnogenol® was as effective in improving symptom scores (Kohama et al., 2007). These studies indicate that this compound could serve as an alternative therapy in the near future. Resveratrol, a compound found in red grapes, also possesses anti-inflammatory properties that have proven promising in alleviating endometriosis-related pain when used in conjunction with OCs (Maia, Haddad, Pinheiro, & Casoy, 2012). Lastly, there is evidence that histone deacetylase inhibitors (HDAC inhibitors), such as trichostatin A (TSA), suppress proinflammatory cytokines such as IL-1β and TNFα (Denk & McMahon, 2012). As mentioned earlier, these play an important role in the inflammatory profile of endometriotic lesions and PF. A study of TSA in a mouse model of endometriosis found that the therapy reduced lesion size and improved response to a painful stimulus (Lu, Nie, Liu, Zheng, & Guo, 2010).

Anti-nociceptive Treatments

Sodium channel blockers are typically prescribed for the treatment of cardiac arrhythmia but have more recently been studied as neuropathic pain blockers as well (Kalso, 2005)**.** Few studies have reported the impact of traditional sodium channel blockers on endometriosis-associated pain, but Wickström and colleagues noted an improvement in the quality of life of patients with endometriosis given lidocaine (Wickstrom, Bruse, Sjosten, Spira, & Edelstam, 2013). Most tri-cyclic antidepressants (TCAs) are also potent sodium channel blockers and, thus, are prescribed for neuropathic pain. The doses at which these drugs can effectively block pain are typically lower than those at which they serve as antidepressants (Hearn, Derry, Phillips, Moore, & Wiffen, 2014), hence the potential for side effects such as drowsiness, constipation,

and blurred vision is lowered. While studies testing the effectiveness of TCAs in treating chronic pelvic pain are scarce, there is a potential to treat the physical symptoms of endometriosis while also improving coping mechanisms.

CONCLUSIONS

There is no shortage of treatments for the various forms of pain associated with endometriosis. While a few options remain first line treatments, it is imperative that we continue to uncover the nociceptive mechanisms behind this condition to help achieve appropriate treatments. In many instances, we can begin working from broader mechanisms such as oxidative stress and inflammation and then narrow it into novel directions. This review provides support for treatments such as antioxidants, medicinal herbs, and antidepressants by addressing novel mechanisms of pain. Figure 1.1 details the mechanisms and non-surgical treatment options, both current and novel, available for endometriosis-associated pain. Table 1.2 summarizes the work that has been completed using these options. The objective of the research detailed in this thesis was to further delve into the afore-mentioned mechanisms of pain. Of particular interest was the role of oxidized stress in endometriosis. Despite the work that has been completed in this area (Table 1.1), a mechanism by which oxidized lipoproteins induce inflammation and nociception has not been elucidated. We hypothesized that these molecules act in a manner similar to prostaglandins and trigger such responses via regulation of epigenetic mechanisms such as microRNAs. Various pathways were investigated using patient tissues as well as animal and cell models of endometriosis.

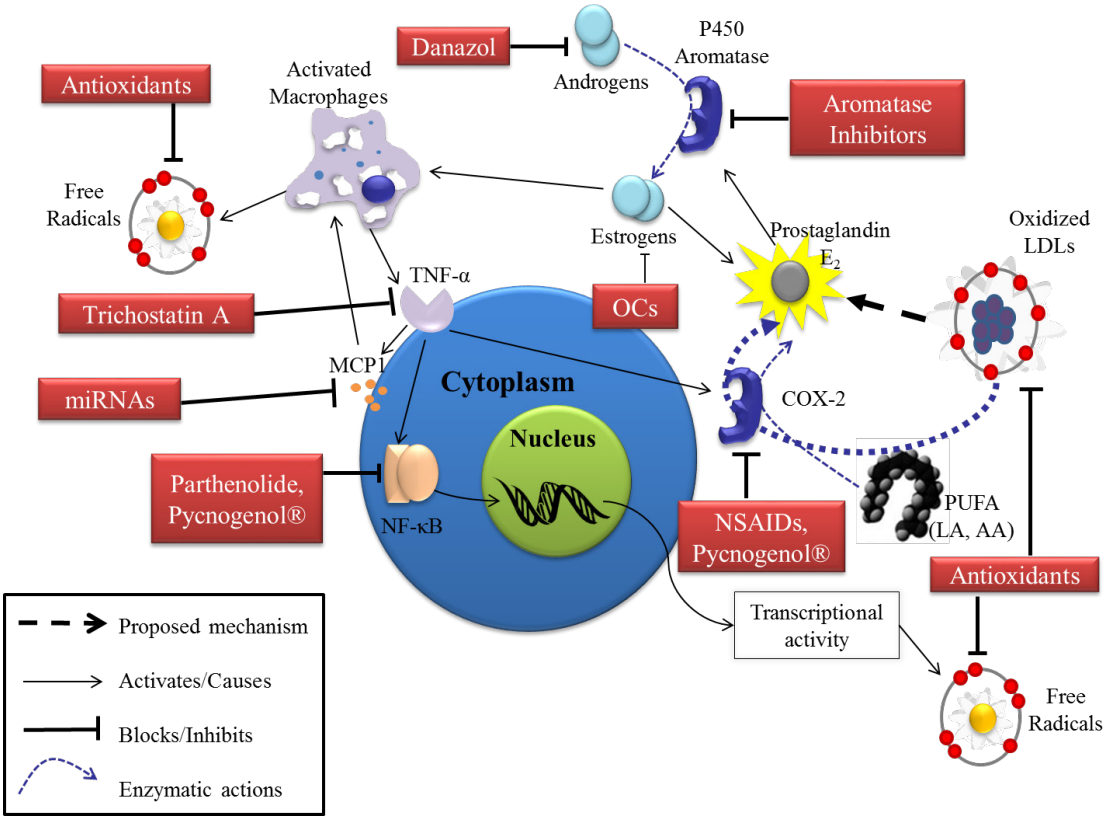

Figure 1.1. New and tested therapeutic targets of endometriosis-associated pain
A general overview of pain mechanisms involved in endometriosis, along with current and potential therapies. COX-2 = cyclooxygenase 2; LDLs = low-density lipoproteins; NF-κB = nuclear factor κB; NSAIDs = nonsteroidal anti-inflammatory drugs; OCs = oral contraceptives; PUFA = polyunsaturated fatty acid (linoleic acid [LA], arachidonic acid [AA]); TNF-α = tumor necrosis factor alpha.

Oxidant	Model	Conclusion(s)	Reference
Macrophages, ROS	Human blood and fluid	Constant stimulation of macrophages in the peritoneal cavity leads to release of ROS	(Zeller, Henig, Radwanska, & Dmowski, 1987)
Nitric oxide (NO)	Human endometrial tissue	There is an overexpression of endothelial nitric oxide synthase in the endometrium throughout the menstrual cycle, suggesting a pathologic role in endometriosis	(Polak, Barczynski, et al., 2013)
Superoxide dismutase (SOD)	Human endometrial tissue	Overexpression of zinc and copper SOD in infertile tissues compared to controls	(Polak, Barczynski, et al., 2013)
MCP-1	Cells (mesothelial and endometrial)	Treatment with ox-LDL led to increased MCP-1, inflammatory action	(Rong et al., 2002)
Oxidative stress and DNA damage	Human tissues	There are significantly higher rates of mtDNA rearrangement and levels of 8-OH-dG, and lipoperoxide contents in endometriotic tissues compared to controls	(Giudice & Kao, 2004)
ROS	Stromal and epithelial primary cells; rodent model	Cells showed an increase in endogenous ROS similar to tumor cells as well as activation of ERK1/2. N-acetyl-cysteine stunted these trends both in vitro and in a mouse model of endometriosis	(Ngo et al., 2009)
Oxidative stress and DNA damage	Human plasma and PF	8-iso-PGF(2α) and oxysterol (the known promoters of steroidogenesis) levels are significantly higher in endometriosis patients than in controls, indicating DNA damage	(Sharma, Dhaliwal, Saha, Sangwan, & Dhawan, 2010)
Antioxidant levels	Human blood serum and PF	Afamin levels are lower in the fluid of women with endometriosis than in controls	(Seeber et al., 2010)
Oxidized liproteins (ox-LDLS)	Human PF	Higher levels of ox-LDLs in the PF of patients with severe endometriosis than patients with serous ovarian cysts	(Polak, Barczynski, et al., 2013)
Copper (Cu) and ceruloplasmin (Cs)	Human	Increased Cu and Cs levels lead to an increase in oxidative stress (as determined by total oxidant status (TOS), oxidative stress index (OSI), triglyceride, cholesterol, and LDL levels)	(Turgut et al., 2013)

Table 1.1. Key investigations into the role of oxidative stress on endometriosis-associated pain

ROS: reactive oxidative species; MCP-1: monocyte chemotactic protein 1; ox-LDL: oxidized low-density lipoprotein; 8-OH-dG: 8-hydroxy-2'-deoxyguanosine; 8-iso-PGF2α: 8-isoprostane-prostaglandin2α

Mechanism	Intervention	Model	Conclusion (s)	Reference
Antioxidants	Vitamins C and E	Human, RCT	Administration of antioxidants (vitamins E and C) reduces chronic pelvic pain in women with endometriosis and inflammatory markers in the peritoneal fluid	(Santanam et al., 2013)
	Melatonin	Human, RCT	Melatonin improves sleep quality and eases endo-associated chronic pelvic pain. It also lowers the levels of brain-derived neurotrophic factor (BDNF) via a mechanism independent of pain.	(Schwertner et al., 2013)
	N-acetylcysteine (NAc)	Human, RCT	NAc can effectively reduce the number of endometriomas, their size, and the pain caused by them. It is a suitable treatment option for women who wish to become pregnant.	(Porpora et al., 2013)
	Chinese herbal medicines (CHMs)	Human, RCT	CHMs did improve pain, quality of life, and self-image scores in 3 separate outcome measures, but results were not significantly different from placebo group (also had herbal treatment). More advance study needed.	(Flower et al., 2011)
Anti-inflammatory	Parthenolide (Anti-NFκB)	Murine	Parthenolide (feverfew) reduces endometrioma growth via blocking the NF-κB pathway	(Takai et al., 2013)
	Pycnogenol® (Anti-COX, Anti- NFκB)	Human, RCT	Pycnogenol® effectievly reduces dysmenorrhea	(Kohama et al., 2007)
	Resveratrol	Human, RCT	Resveratrol serves as an effective adjuvant endo pain treatment to oral contraceptives by helping to block aromatase and COX-2	(Maia et al., 2012)
	HDAC Inhibitors (TSA)	Murine	HDAC Inhibitors are effective in inflammatory disease and can block inflammatory effects of IL-1β and TNFα	(Y. L. Chung, Lee, Wang, & Yao, 2003; Glauben et al., 2006; Leoni et al., 2005)
Sodium channel blockers	Lidocaine	Human, RCT	Pertubations with lidocaine increased the quality of life (scores) of endo patients	(Wickstrom et al., 2013)
	TCAs (Imipramine)	Review (proposed)	Currently prescribed for neuropathic pain	(Hearn et al., 2014)

Table 1.2. Novel and proposed therapies for endometriosis-associated pain. RCT: randomized controlled trial; NFκB: nuclear factor κB; COX: cyclooxygenase; TCAs: tricyclic antidepressants; HDAC: histone deacetylase; TSA: trichostatin A

CHAPTER 2

OXIDATION SENSITIVE NOCICEPTION INVOLVED IN ENDOMETRIOSIS ASSOCIATED PAIN

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ABSTRACT

Endometriosis is a disease characterized by the growth of endometrial tissue outside the uterus and is associated with chronic pelvic pain. Peritoneal fluid (PF) of women with endometriosis is a dynamic milieu, rich in inflammatory markers and pain-inducing prostaglandins $PGE_2/PGF_{2\alpha}$ and lipid peroxides, and the endometriotic tissue is innervated with nociceptors. Our clinical study showed the abundance of oxidatively-modified lipoproteins in the PF of women with endometriosis and the ability of antioxidant supplementation to alleviate endometriosis-associated pain. We hypothesized that oxidatively-modified lipoproteins present in the PF are the major source of nociceptive molecules that play a key role in endometriosis-associated pain. In this study, PF obtained from women with endometriosis or control women were used for (i) the detection of lipoprotein derived oxidation-sensitive pain molecules, (ii) the

ability of such molecules to induce nociception, and (iii) the ability of antioxidants to suppress this nociception. LC-MS/MS showed the generation of eicosanoids by oxidized-lipoproteins similar to that seen in the PF. The oxidatively-modified lipoproteins induced hypothermia (intra-cerebroventricular) in CD-1 mice and nociception in the Hargreaves paw-withdrawal latency assay in Sprague-Dawley rats. Antioxidants, vitamin-E and N-acetylcysteine and the NSAID, indomethacin suppressed the pain inducing ability of oxidatively-modified lipoproteins. Treatment of human endometrial cells with oxidatively-modified lipoproteins or PF from women with endometriosis showed up-regulation of similar genes belonging to the opioid and inflammatory pathways. Our finding that oxidatively-modified lipoproteins can induce nociception has a broader impact not only in the treatment of endometriosis-associated pain but also in other diseases associated with chronic pain.

INTRODUCTION

Endometriosis is a highly debilitating inflammatory disease (Lousse et al., 2012) that afflicts 10-15% of women of child-bearing age (Augoulea, Alexandrou, Creatsa, Vrachnis, & Lambrinoudaki, 2012; Burney & Giudice, 2012). It is characterized by the presence of endometrial cells outside the uterus and often presents with pain and/or infertility. Endometriosis is most commonly associated with dysmenorrhea, dyspareunia, non-cyclic pain and abdominal pain (Stratton & Berkley, 2011; Taylor, Hummelshoj, Stratton, & Vercellini, 2012). Laparoscopic surgery to remove the endometriotic tissue is a major treatment option to relieve pain, but often results in recurrence of the disease (Deguara, Pepas, & Davis, 2012; Koga, Osuga, Takemura, Takamura, & Taketani, 2013). The relationship between the severity of the disease and the presence of pain symptoms is not well defined (Milingos et al., 2006). It has been hypothesized that the ectopic lesion releases chemotactic molecules that attract immune cells into

the peritoneal cavity, accumulating in the peritoneal fluid (PF) (Bedaiwy & Falcone, 2003; Kyama et al., 2009). This triggers the secretion of more cytokines and growth factors, thus sustaining the growth of the lesion (Barcz, Kaminski, & Marianowski, 2000).

The cyclooxygenases (COX-1 and COX-2) and 12, 15, or 5-lipoxygenase derived lipid-mediators from arachidonic acid (AA), such as prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ (Petho & Reeh, 2012) and 12- and 15-(*S*)-hydroperoxyeicosatetraenoic acids (HPETE), 5- and 15-(*S*)-hydroxyeicosatetroenoic acids (HETE), and leukotriene B4 are potent activators of nociceptors (Durand et al., 2009; Smith, 2006) and participate in nociception, or the ability to feel pain (Zeilhofer, 2007). Endometriotic tissue expresses COX-2 (Buchweitz et al., 2006; Lousse, Defrere, Colette, Van Langendonckt, & Donnez, 2010) and PF of patients with endometriosis contain varying amounts of PGE_2 and $PGF_{2\alpha}$ (Sacco, Portelli, Pollacco, Schembri-Wismayer, & Calleja-Agius, 2012; M. H. Wu et al., 2010). Chronic pain is often attributed to tissue inflammation and injury resulting from oxidative stress and oxidants such as superoxide and nitric oxide (NO) play a role in nociception (Aley, McCarter, & Levine, 1998; Z. Q. Wang et al., 2004).

Non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit COX enzymes and prevent the enzymatic oxidation of AA to generate prostanoids are the most commonly prescribed agents to alleviate pain in endometriosis (Allen et al., 2009; Streuli et al., 2013). Interestingly, AA can undergo non-enzymatic (free-radical mediated) oxidation to generate prostaglandin-like products that may not be inactivated by NSAIDs (Durand et al., 2009). The source or nature of these prostaglandin-like products, the likelihood of these molecules to play a role in nociception and agents that inhibit their production is not currently known. This discovery will be highly beneficial in the treatment of chronic pain.

Studies from our laboratory have shown the importance of oxidative stress in the etiology of endometriosis (Murphy, Santanam, Morales, & Parthasarathy, 1998; Murphy, Santanam, & Parthasarathy, 1998; Santanam, Murphy, et al., 2002) and showed increased presence of oxidative and inflammatory stress markers in the PF of women with endometriosis (Santanam, Murphy, et al., 2002). Many of these oxidant-sensitive markers increase inflammatory response and endometriotic lesion growth in animal models of endometriosis (Carvalho et al., 2012; Murphy, Santanam, & Parthasarathy, 1998), which can be prevented by antioxidant (N-acetylcysteine or vitamin E) supplementation (Pittaluga et al., 2010; Porpora et al., 2013; Santanam et al., 2013). Interestingly, our clinical trial showed that antioxidant (vitamin E and C) supplementation also lowered pain responses in women with endometriosis (Santanam et al., 2013) thus suggesting that the nociceptive molecules are oxidation-sensitive. We have shown that women with endometriosis have high levels of lipoproteins (abundant in AA), in their PF (Murphy, Santanam, Morales, et al., 1998; Murphy, Santanam, & Parthasarathy, 1998; Santanam, Murphy, et al., 2002). In the present study, we provide evidence that these lipoproteins undergo non-enzymatic oxidation and generate prostaglandin-like molecules that modulate nociception in animal models of pain. Antioxidants can suppress the generation of these nociceptive molecules.

MATERIALS AND METHODS

Human Subject Participants

Approximately, 50 women/group, ages 18-60 years undergoing tubal ligation (control women, without endometriosis) or undergoing laparoscopy for endometriosis were recruited from the Department of Obstetrics and Gynecology, Cabell Huntington Hospital, Marshall University School of Medicine, Huntington, WV and Emory University School of Medicine,

Atlanta, GA. This HIPAA compliant study was approved by the Institutional Review Board of the Marshall University School of Medicine and Emory University School of Medicine and was carried out according to the principles of the Declaration of Helsinki. All patients were consented prior to the study. All women completed a validated patient history form and an assessment form of pain using a visual analogue scale for assessment of endometriosis associated pain (dysmenorrhea, non-menstrual pelvic pain, dyspareunia, and dyschesia) (adapted from the validated International Pelvic Pain Society's Pelvic Assessment Form). Peritoneal fluid (devoid of blood contamination) was collected on ice during surgery, then immediately transferred to the research facility and centrifuged at 2000xg to remove any cellular debris. The supernatant was used immediately for studies or stored at -80^{0}C freezer for future use. The inclusion criteria for the study included adult non-smoking women, age 18-60 years, with normal menstrual cycles and otherwise in normal health (except for pain and endometriosis) who had not been on any hormonal medication for at least 1 month before sample collection. Exclusion criteria included subjects with current medical illnesses such as diabetes, cardiovascular disease, hyperlipidemia, hypertension, systemic lupus erythematosis or rheumatologic disease, positive HIV/AIDS, active infection, current medications such as hormonal/anti-hormonal medications, anti-inflammatory medications including corticosteroids. Subjects were asked to stop multivitamins that contain high levels of antioxidants at least one week prior to the surgery. The menstrual phase was calculated from the last menstrual period data obtained from the patient history forms.

Low Density Lipoprotein (LDL) Isolation.

LDL was isolated from heparinized blood obtained from normal human volunteers by the single-spin ultracentrifugation technique using a Beckman Table Top TL100 ultracentrifuge (B. H. Chung, Wilkinson, Geer, & Segrest, 1980). Since lipo-polysaccharide (LPS) contaminations

can induce inflammatory responses, careful precautions were used to avoid any auto-oxidation or LPS contamination. LPS free water and buffers were used for all isolation procedures and the LPS levels were determined using the LAL assay (Limulus Amoebocyte Lysate assay, CapeCod Inc, Falmouth, MA). The protein concentration of the isolated LDL was measured using Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951).

Oxidation of LDL in the Presence or Absence of Antioxidants or NSAIDs

Briefly, 100µg/mL of LDL was incubated with 5µM of copper sulfate (an oxidant) in 1mL of 1X phosphate buffered saline (PBS, pH 7.4). Lipid peroxidation was initiated by the reaction between copper and polyunsaturated lipids present in the lipoproteins. The oxidation was measured in a Shimadzu UV-VIS spectrophotometer by following the generation of conjugated-diene products (lipid peroxidation marker) which has a unique absorption at OD 234nm (Supplementary Figure 2.1A). The oxidation process was terminated at specific time points to generate various forms of oxidatively-modified LDL preparations, by the addition of 50µM of 1mM EDTA (a copper chelator). The LDL preparations included: (a) native LDL (L0), (b) minimally-modified LDL-L1 (usually terminated at the end of the lag time), (c) oxidized LDL-L2 (after the oxidation has reached its plateau) and (d) completely or fully oxidized LDL-L3 (after 24 hours of oxidation) (Parthasarathy, Auge, & Santanam, 1998; Parthasarathy, Raghavamenon, Garelnabi, & Santanam, 2010; Parthasarathy, Santanam, & Auge, 1998). Control LDL preparations were incubated without copper and stopped at various time points similar to the one that goes through oxidation (in the presence of copper) to serve as respective non-oxidized LDL controls. These various forms of LDL preparations represent LDL that has undergone oxidation at various levels and thus has undergone changes in both chemical and biochemical properties (Parthasarathy, Auge, et al., 1998; Parthasarathy et al., 2010;

Parthasarathy, Santanam, et al., 1998; Parthasarathy, Santanam, Ramachandran, & Meilhac, 2000). Oxidative modification of LDL increases its negative charge which is reflected by its increased electrophoretic mobility as shown in Supplementary Figure 2.1B (Arrio, Bonnefont-Rousselot, Catudioc, & Packer, 1993). These represent heterogeneous LDL molecules that are theoretically possible to be physiologically present (Parthasarathy et al., 2000). The various LDL preparations were prepared fresh for use in nociception and all other studies.

For antioxidant studies, the above LDL preparations were generated in the presence of antioxidants, N-acetylcysteine (1mM) or vitamin E (50µM) or the COX inhibitor indomethacin (1µg) (NSAID). Briefly, 100µg/mL of LDL was incubated with 5µM of copper sulfate in the presence of these agents and the oxidation was followed at OD 234 nm in a UV-VIS spectrophotometer. These preparations were used in the cell culture experiments and in Hargreaves nociception assay.

Thiobarbituric Acid Related Substances (TBARS)

TBARS are a measure of the extent of oxidation when lipids undergo peroxidation. TBARS were measured in all the LDL preparations at the end of oxidation, as an increase in optical density 540nm using a standardized protocol (Yagi, 1998). The amounts of TBARS were quantitated using commercially available malondialdehyde (Sigma-Aldrich, St. Louis, MO) as standard and expressed as nmoles/mg protein. Typically, TBARS levels of the various preparations range as follows: L0<5 nmoles; L1=15-20 nmoles; L2=35-40 nmoles; L3>35 nmoles.

Agarose and Native Gel Electrophoresis for Lipoproteins

Agarose gel electrophoresis (Beckman Coulter Inc., Brea, CA) was used to separate lipoproteins in the PF and plasma and the various oxidatively modified LDL preparations (L0,

L1, L2 and L3) using manufacturer's instructions. The lipoproteins were identified using Fat red O staining. Commercially available LDL and HDL samples were used as standards. 10% native polyacrylamide gel electrophoresis was used to detect the presence of apolipoprotein B (Mol weight >200 kd) in the PF samples. Proteins were detected using Coomassie brilliant blue after separation of the samples on a native PAGE.

Prostaglandin E_2 (PGE_2) and 8-Isoprostane (8-Iso) Detection Using EIA Kits

In order to assess if the non-enzymatic oxidation of lipoproteins can generate PGE_2-like molecules, PGE_2 and 8-isoprostane levels were detected in both the PF and oxidatively modified LDL preparations using commercially available Enzyme Immunoassay (EIA) kits from Cayman Chemicals (Ann Arbor, MI). 100µl of the LDL preparations were either directly (no dilution) or after dilutions (10-1000 fold dilution in 1xPBS) and 50 µL of PF were used in the EIA assays. Manufacturer's instructions were followed for both the measurements and post-analysis of PGE_2 and 8-isoprostanes and expressed as pg/ml. T-test was used to compare the levels of PGE_2 in the control PF to the levels in endometriotic PF. In addition, one-way ANOVA followed by Dunnett's multiple comparison test was used to compare the levels of PGE_2-like molecules generated in all the oxidatively modified LDL preparations (L1, L2, L3) to the levels generated in the native LDL (unoxidized LDL-L0) control preparation.

LC-MS/MS Detection

Eicosanoids and 20:4n6 lipids were extracted from PF and LDL preparations using an acetone liquid/liquid extraction (Brose & Golovko, 2013; Brose, Thuen, & Golovko, 2011; Golovko & Murphy, 2008) with slight modifications. PF or LDL preparations (500µl) was extracted with 500µl saline (0.9% NaCl) and 2mL acetone with deuterium labelled internal standards (100pg PGE_2-d_9; 500pg $PGF_{2\alpha}$-d_4, LTB_4-d_4, TxB_2-d_4, 5S-HETE-d_8, 12S-HETE-d_8;

10ng 20:4n6-d_8) (Cayman Chemicals) at pH 3.0 followed with 2 mL of chloroform with 0.005% BHT. The extract was dried under nitrogen and re-dissolved in 12µl of acetonitrile:water (40:60). The LC-MS/MS system consisted of an ACUITY UPLC pump (Waters; Milford, MA), and a XEVO TQ-S triple quadruple mass spectrometer (Waters) with electrospray ion source. The autosampler temperature was 8°C. Ten µL of sample was injected onto an ACUITY UPLC HSS T3 column (1.8µM, 100Å pore diameter, 2.1×150 mm, Waters) with an ACUITY UPLC HSS T3 precolumn (1.8µM, 100Å pore diameter, 2.1×5 mm, Waters). The separation was performed as previously described (Brose, Baker, & Golovko, 2013; Brose & Golovko, 2013). The flow rate was 0.45mL/min and the initial conditions were 39%B (0.1% formic acid in acetonitrile) and 61%A (0.1% formic acid in water). At 0.5min solvent B was increased to 40.5% over 6.88min, then increased to 70% over 1.62min, then increased to 75% over 3min, further increased to 98% over 1.5min and held for 5.3min. The solvents were then returned to initial conditions over 0.2min and held for 2min to re-equilibrate the column. The mass spectrometer was operated in negative ion mode. The capillary and cone voltage were 2.3kV and 30V, respectively. The desolvation and source temperature were 550C and 150C, respectively. The nebulizer gas was 7.0 bar and the desolvation and cone gas flows were 1000L/h and 150L/h, respectively. MassLynx V4.1 (Waters) was used for instrument control, data acquisition and sample analysis.

$PGF_2\alpha$, TxB_2, 20:4n6, LTB_4, and 5-HETE were quantified using $PGF_2\alpha$-d_4, TxB_2-d_4, 20:4n6-d_8, LTB_4-d_4, and 5S-HETE-d_8 as an internal standard, respectively, 12-HETE and 15-HETE using 12S-HETE-d_8, and all other PG were quantified using PGE_2-d_9 as previously validated (Golovko & Murphy, 2008). Analytes were monitored in MRM mode as previously described (Brose & Golovko, 2012, 2013; Brose et al., 2011; Golovko & Murphy, 2008) using

the following mass transitions: PGE_2-351.18/271.13; 11β-PGE_2-351.18/271.13, 8-*iso*PGE_2-351.18/271.13, PGD_2-351.06/271.14; 6-keto$PGF_{1\alpha}$-369.26/163.07; $PGF_{2\alpha}$-353.07/193.04; TXB_2-369.20/169.00; 20:4n6-303.07/259.21; LTB_4-335.07/194.99; 5-HETE-319.20/115.20; 12-HETE-319.10/267.21; 15-HETE - 319.12/219.09; PGE_2-d_9-360.2042/280.17; $PGF_{2\alpha}$-d_4-357.16-197.01; TXB_2-d_4-373.22-173.03; 20:4n6–d_8-310.93/267.21; LTB_4-d_4-339.26/197.06; 5S-HETE-d_8-326.86/116.03; 12S-and-HETE-d_8-327.12/184.04. The collision energies used were (eV): PGE_2-16; PGD_2-16; 11β-PGE_2-16, 8-*iso*PGE_2-16,6-keto$PGF_{1\alpha}$ - 24; $PGF_{2\alpha}$-20; $PGF_{2\alpha}$-d_4-22; TXB_2-12; PGE_2-d_9-14; TXB_2-d_4-12; 20:4n6-12; LTB_4-14; 20:4n6-d_8-12; LTB_4-d_4-14; 5-HETE-10; 12-HETE-12; 15-HETE-10; 5S-HETE-d_8-14; 12S-HETE-d_8-12.

Human Pain: Neuropathic and Inflammatory RT^2 PCR Array

Ishikawa cells (Sigma, St. Louis, MO), a human (39 year old female) established endometrial cell-line, was cultured in T75 flasks in complete media (DMEM/F12, Pen/Strep, FBS, glutamine). These cells were used since they express similar characteristics of mature endometrial epithelial cells (Castelbaum et al., 1997; Lessey et al., 1996; Sugihara et al., 2014). About 80% confluent cells were treated with either PGE_2 (50ng/ml), 25µg of various LDL preparations, or 100µl of PF from patients with and without endometriosis for 48 hours. The concentrations chosen were selected from preliminary unpublished studies. At the end of 48 hours, cells were collected using Qiazol reagent (Qiagen, Gaithersburg, MD) and RNA was isolated using Qiagen RNeasy Mini Kit. cDNA synthesis from 1µg of each sample was achieved using Qiagen RT^2 First Strand Kit. Nociceptive and inflammatory pathway genes were analyzed in the cDNA samples using the commercial Human Pain: Neuropathic and Inflammatory RT^2 PCR Array (PAHS-162ZA, Qiagen, Valencia, CA) on the Biorad MyiQ system. Ishikawa cells treated with 1% charcoal-stripped serum containing media alone (DMEM/F12, Pen/Strep,

charcoal-stripped FBS, glutamine) were used as the control group. Fold change was determined using Pfaffl equation [$2^{\wedge-(ddct)}$] for all groups compared to media control using the manufacturers (Qiagen) algorithm which uses T-test as the default statistics to compare differences between control and treated groups. A stringent 4-fold cutoff was used to identify differentially expressed genes in Ishikawa cells treated by various groups compared to the charcoal-stripped media treated cells (control group).

Body Temperature Assay (Intracerebroventricular Injections-Hypothermia/Hyperthermia)

Intracerebroventricular (i.c.v.) injection of PGE_2 produces fever (hyperthermia) through an agonist action at the four subtypes of EP receptors (notably EP3 and EP1). Prostaglandins interact with these receptors to modulate body temperature (hypothermic or hyperthermic response) (Furuya et al., 2003; Oka, 2004). To assess whether the oxidized LDL preparations function similarly, we assessed the effect of these lipids on body temperature in mouse models. An IACUC approval from Louisiana Health Sciences Center Institutional Review committee was obtained for this study. All investigators were certified to perform animal studies. Groups of male CD-1 mice (n =8; Charles River, Boston, MA) weighing 34-40g were acclimated to the testing room for 3 hrs. Baseline body temperatures were taken rectally three times separated by at least 10 min using a thermistor telethermometer (Cole-Parmer). Only the last determination was used for statistical comparison. After baseline testing, 5 µl of saline (control), 1ng/ml PGE_2, LDL-L0 (100 µg/ml), or one of the oxidized LDL preparations (L1, L2, L3) was injected i.c.v. using a 30 gauge needle attached to a Hamilton microsyringe with PE10 tubing (Haley & McCormick, 1957). After injection, body temperature was assessed every 10 min for 1 hr. After the first hour, temperatures were assessed at 120 min, 160 min and 24 hours after initial injection. Baseline temperatures were subtracted from post-injection temperatures, and reported

as mean change in body temperature. The mice were euthanized at the end of the study using IACUC approved procedures. Two-way ANOVA (repeated measures) followed by Bonferroni's post-hoc test was used to assess the difference in body temperature between saline injection and the other treatments over time.

Hargreaves Paw Withdrawal Pain Assay

To assess whether the non-enzymatically oxidized lipoproteins are a potential source of pain inducing molecules, we performed a Hargreaves assay of paw withdrawal latency as a measure of in vivo pain response using known pain inducers (carrageenan and PGE2), and compared it to native LDL (L0) and its oxidized forms (L1 and L2). The Hargreaves Method (Hargreaves, Dubner, Brown, Flores, & Joris, 1988) was performed to measure nociception (pain) in rodent models using the IITC Model 390 Plantar Test Analgesia Meter (Woodland Hills, CA). An IACUC approval from Marshall University Institutional Review committee was obtained for this study. All investigators were certified to perform animal studies. Male Sprague-Dawley rats (Hill-top Lab animals, Scottdale, PA), 7-8 weeks of age, were used for this study. Briefly, a beam of light was directed onto the midplantar (dorsal surface) region of the hindpaws. The operator turned off the light and recorded the time of withdrawal of the hindpaw from the surface. The active intensity was set at 25% and the cutoff time as 20 secs. Treatments were performed through a randomized blinded study. Each treatment sample (100µL) was injected into the dorsal surface of the hindpaw of the rats and readings (paw withdrawal time) were measured every 30 minutes for the first hour and subsequently every hour for a total of 8 hours. An additional measurement was performed after 24 hours, post-injection. Saline injected on the left paw was used as an internal control to account for volume and pain related behavior associated with injection. Treatments included 3% carrageenan (an irritant that produces pain),

50ng/mL PGE_2, 100µl of different oxidatively modified forms of LDL protein (100µg/mL), PF from women with and without endometriosis (100µL). Carrageenan served as a positive control to validate the use of the Hargreaves paw withdrawal pain assay. In order to investigate if antioxidants would mitigate the nociceptive response mediated by oxidatively-modified lipoproteins, LDLs prepared in the presence of antioxidants 1mM N-acetylcysteine, and 50µM vitamin E or the COX inhibitor 1µg indomethacin were also tested for nociception. The paw-withdrawal time was recorded in triplicates for each treatment per time point per rat. The number of rats used for each experiment varied according to the treatment and were kept at least n>6 for all treatments. The rats were euthanized at the end of the pain assessment using IACUC approved procedures. The data were averaged for each time point and % withdrawal latency was determined by comparing the treatment response time versus saline response time (Equation 1). Thus, % withdrawal latency <100 is indicative of increased pain related behavior associated with the stated treatment, whereas a % withdrawal latency >100 is indicative of a decrease in pain related behavior with the stated treatment.

Equation 1. *% Withdrawal Latency = [average time response to paw withdrawal of treatment]/[average time response to paw withdrawal of saline]*

Statistical Analysis

Prism© software (Graphpad, Inc., La Jolla, CA) was used for statistical analysis of human and cell culture studies. T-test was used for detecting differences in eicosanoids (LC-MS), prostaglandins, 8-isoprostanes (EIA) and electrophoretic mobilities of isolated lipoproteins between control and endometriotic PF. One-way ANOVA followed by Dunnett's multiple comparison test was used to find differences in PGE_2 levels between unoxidized LDL (L0) and the oxidized preparations (L1-L3). Two way ANOVA followed by Bonferroni's post hoc test

was used to detect differences in body temperature (icv) between treatments and saline (control) over time.

Statistical analysis on results generated by the Hargreaves pain assay was implemented in R (v 3.0.2) (www.r-project.org). Significance was defined at p-values < 0.05 (two-sided). Linear mixed effects (LME) models on log10 transformed data were used to identify significant differences in pain induction between the treatment groups of interest and the internal control (saline) through time. All models originally contained an interaction term with the time. Paired t-tests were used to compare differences between treatment and saline at each time as a follow-up exploratory analysis following LME; however, it should be stated that the use of the paired t-test may increase probability of type 1 error.

Prism© software (Graphpad, Inc., La Jolla, CA) was used for preparation of graphs.

RESULTS

Human Studies

Presence of Cyclooxygenase and Lipoxygenase Generated Eicosanoids in PF of Women with Endometriosis

EIA analysis was done on the PF samples from endometriosis and control subjects. There was an increased presence of enzymatically derived PGE_2 (p=0.07) (Figure 2.1A) and free-radical or non-enzymatically derived 8-isoprostanes (p=0.005, T-test) (Figure 2.1B) in the PF of women with endometriosis (n=43) compared to control (n=36) subjects. LC-MS/MS separation of the PF from a representative number of patients (n=6/group) was done to confirm the presence of eicosanoids (both enzymatic and non-enzymatically generated). Figure 2.1C showed higher levels of 12, 15 or 5-lipoxygenase derived eicosanoids such as 12, 15-HETEs (p<0.05, T-test) or

5-HETEs, and though not significant, cyclooxygenase derived eicosanoids such as PGE_2 and PGD_2 in endometriosis patients compared to control subjects.

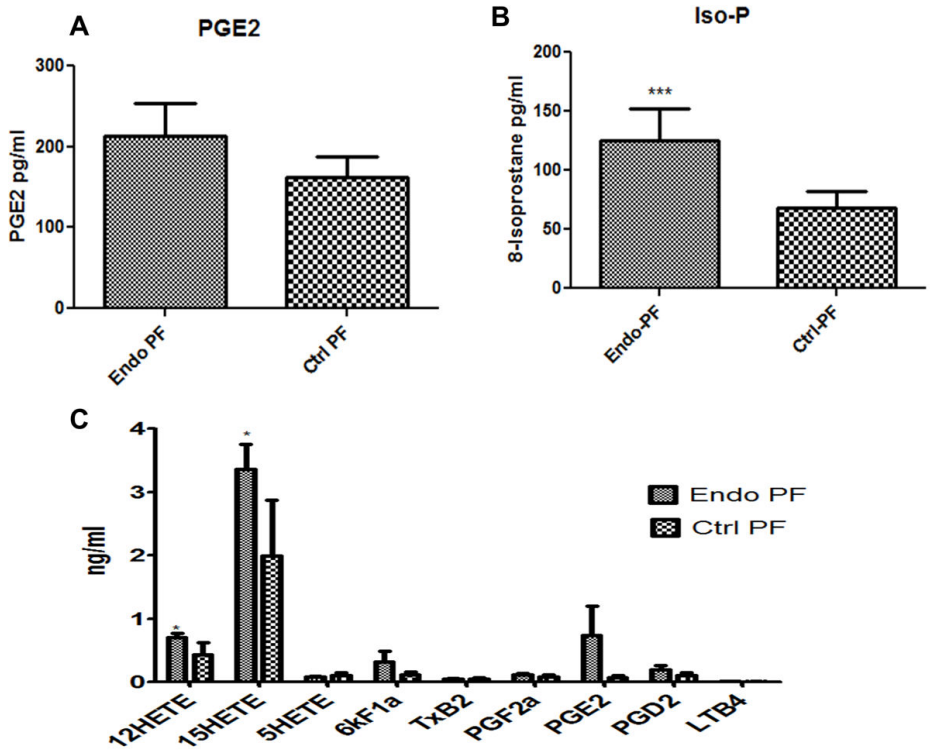


Figure 2.1. Presence of eicosanoids in peritoneal fluid. (A, B) Enzyme Immunoassay-EIA showed the presence of COX generated PGE_2 and free-radical generated 8-isoprostanes in the PF. The levels of PGE_2 ($p<0.07$) and 8-isoprostanes ($p<0.005$) were higher in the PF from endometriosis patients (n=43) compared to control women (n=36). Results are expressed as pg/ml ± SEM. (C) LC-MS/MS separation of PF lipids showed the presence of higher levels of both COX generated prostanoids (PGE_2, PGD_2) as well as 12, 15, 5-LOX generated 12, 15, 5-HETEs in the PF from endometriosis (n=6) patients compared to control women (n=6). T-test was used for statistical analysis. $P<0.05$ was considered significant.

Evidence for Presence of Modified Lipoproteins in the PF of Women with Endometriosis.

Supplementary Figure 2.2A shows a typical agarose gel separation of lipoproteins (LDL, VLDL and HDL) present in plasma (PL), LDL isolated from the plasma (Pl-LDL), peritoneal fluid (PF) and LDL isolated from peritoneal fluid (PF-LDL). As shown earlier, oxidative modification of LDL increases its negative charge which is reflected by its increased

electrophoretic mobility (Supplementary Figure 2.1B) (Arrio et al., 1993). We have earlier shown that while 90% of the PF samples from subjects with endometriosis were positive for the presence of lipoproteins (LDL and HDL), only 45% from normal subjects were positive for lipoproteins (Murphy, Santanam, Morales, et al., 1998; Murphy, Santanam, & Parthasarathy, 1998). We had also previously shown that plasma LDL and PF-LDL of subjects with endometriosis had significantly greater electrophoretic mobilities of 0.87±0.08 and 1.37±0.15cm, respectively ($p<0.005$, T-test) compared to 0.6±0.02 and 0.8±0.06cm in control subjects, respectively (Murphy, Santanam, Morales, et al., 1998; Murphy, Santanam, & Parthasarathy, 1998). The presence of lipoproteins in the PF of subjects with endometriosis did not appear to be due to contamination by blood. Supplementary Figure 2.2B showed an intact apolipoprotein B (apoB) in the LDL isolated from the PF.

Non-enzymatic Oxidation of LDL Generates Prostaglandin-like Molecules

Figure 2.2A shows that the non-enzymatically modified LDL preparations generated PGE_2-like molecules measurable using PGE_2 Enzyme Immunoassay (EIA) kits (Cayman chemicals, Ann Arbor, MI). Compared to native LDL (L0), the levels of PGE_2-like molecules increased with the extent of LDL oxidation, (2-3 fold=L1, 5-6 fold=L2 and 10-12 fold=L3) ($p<0.0001$, one-way ANOVA, Dunnett's multiple comparison test), i.e. the more the length of oxidation time, the more formation of PGE_2-like molecules were observed. LC-MS/MS studies confirmed the generation of prostanoids PGE_2, PGD_2 and 11β-PGE_2 and lipoxygenase products 12, 15 and 5-HETEs in the non-enzymatically modified LDLs (Figure 2.2B). The prostanoid levels increased whereas LOX products decreased with increase in extent of LDL oxidation (Figure 2.2C).

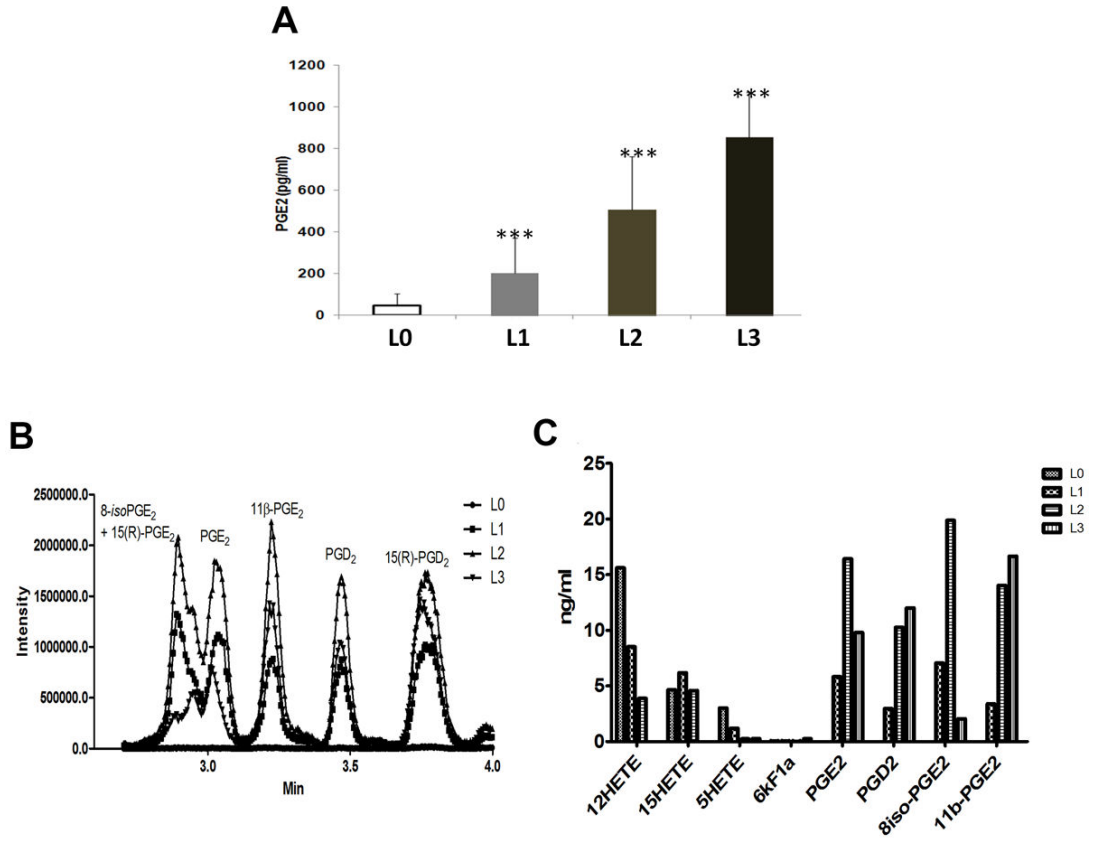


Figure 2.2. Detection of prostaglandin-like molecules by LC-MS/MS. (A) Oxidatively-modified LDL preparations were used to detect the presence of prostaglandin-like molecules using PGE2 EIA kit. The levels of prostaglandin-like molecules as measured by EIA increased as the level of oxidation of LDL increased, with L0 (control group) having the least level compared to L3 (n=10-12). (B and C) LC-MS/MS was used to detect eicosanoids generated by non-enzymatic oxidation of LDLs. Both enzymatic (PGE2, PGD2), and non-enzymatically generated oxidation products were generated during the oxidation of lipoproteins. One-way ANOVA followed by Dunnetts' multiple comparison test was used for statistical analysis. $P<0.05$ was considered significant.

Studies in Rodent Model

ICV Injections of LDL in Mouse Model Induce Thermal Response

Intracerebroventricular (i.c.v.) injection of PGE_2 (Figure 2.3), produced a significant (Two way ANOVA, Bonferroni posttest) increase in body temperature (38^0 to 40^0) within 30 minutes ($p<0.001$) of injection compared to saline injection. This response lasted until 60-90 minutes ($p<0.01$ at 90 min). In contrast, L0 (native LDL) and its three oxidatively modified LDLs (L1-L3) all produced an initial drop (hypothermia) in body temperature (-2 to -4 degree

Celsius, 38^0 to 35^0) around 30-40 minutes. L0 and L3 injected mice returned to baseline temperatures (38^0C) within 90 minutes; however, the L1 and L2 injected mice maintained higher temperature for more than 100-120 minutes ($p<0.05$ for L1 and $p<0.01$ for L2 at 120 min and $p<0.01$ for L2 at 160 min). These results are indicative of these lipids functioning similar to prostanoids (PGE_2) in modulating body temperature either through EP receptors or others such as opioid receptors (Furuya et al., 2003).

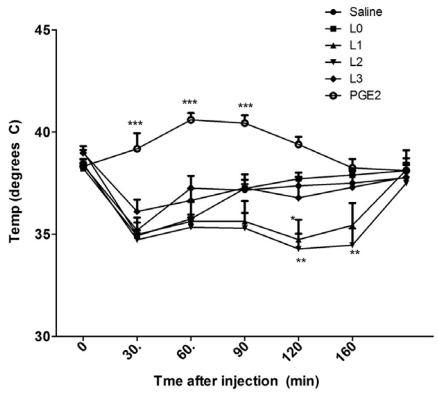


Figure 2.3. Oxidatively-modified LDLs and body temperature. Baseline rectal temperatures were assessed in five groups of CD-1 mice (n=10). Mice were then injected i.c.v. with native LDL (L0), minimally modified LDL (L1), oxidized LDL (L2), fully oxidized LDL (L3), or PGE_2 and temperature assessed every ten min for sixty min and recorded as a difference from baseline (0 = 38.4ºC, SEM = 0.2). PGE_2 produced a significant increase in body temperature (38^0 to 42^0C) from 20 min to 50 min after injection, whereas native LDL (L0) and its oxidized preparations (L1, L2, L3) produced a decrease in body temperature (38^0 to 35^0) from 10 min through 60 min. Two-way ANOVA followed by Bonferroni posttest was used to assess differences in body temperature between saline injection and other treatments over time.

Oxidatively-modified Lipoproteins Induce Pain Related Behavior

In the Hargreaves paw withdrawal assay, all treatments (carrageenan, PGE_2, LDL preparations) injected on the dorsal surface of the right paw of SD rats were compared against the respective internal saline control (injected on the dorsal surface of the left paw). A paw withdrawal latency <100% indicates an increase in pain related behavior induced by the stated treatment. Figure 2.4A illustrates a typical % withdrawal latency ± SEM (as compared to saline)

curve following injection with known pain inducers, carrageenan or PGE_2. Carrageenan, an irritant (n=5) served as a positive control to validate the use of the Hargreaves pain assay. Compared to saline, significant reductions in withdrawal latencies (indicative of increased pain sensitivity) were observed at 60min, 180min and 240min post-injection with carrageenan (p: 0.013, 0.043 and 0.002, respectively). Compared to saline, treatment with 50ng/mL PGE_2 (n=22) induced significant reductions in withdrawal latency at 30min, 60min, 120min, and 180min post-injection (p: 0.0002, 0.011, 0.025, and 0.0008 respectively); however, no differences in withdrawal latency were observed beyond 180min. Figure 2.4B illustrates the % withdrawal latency ± SEM (as compared to saline) following injection with native-LDL (L0; n=29), minimally modified-LDL (L1; n=44) or oxidized-LDL (L2; n=37). Compared to saline, treatment with L1 induced significant reductions in withdrawal latency at 240min (p: 0.0008) and L2 at 60min, 240min, 300min and 360min post-injection (p: 0.005, 0.045, 0.014, and 0.0472, respectively). L0 indicated a reduction in withdrawal latency at 120min compared to saline.

Raw paw withdrawal response times (seconds) for each treatment group and the respective saline control over the course of 8 hours is provided in Supplementary Figure 2.3. Statistical analysis using LME models comparing treatments to saline (internal control) and with "time" as the interaction term, showed no significant interaction for saline versus treatment and time for Carrageenan, L0 and L2. However, comparing PGE_2 to saline showed a significant interaction (p: 0.0002) between time and treatment versus saline indicating a different time course of pain related behavior for PGE_2 versus saline. Similarly, comparing L1 to saline, showed a significant interaction (p: 0.0039) indicative of a different time course of pain related behavior for L1 versus saline.

Peritoneal Fluid from Women with Endometriosis Induces Pain Related Behavior

Figure 2.4C illustrates the % withdrawal latency ± SEM (as compared to saline) following injection with PF from women with endometriosis (+Endo; n=8 subjects, in quadruplicates) or women without endometriosis (-Endo; n=8, in triplicates). Compared to saline, treatment with PF from women with endometriosis (+Endo) indicated significant reductions in withdrawal latency (i.e. increased pain related behavior) at 30min (p: 0.011) (time at which PGE_2 induces pain related behavior, indicated by an arrow in the figure), as well as at 240min and 300min post-injection (p: 0.003 and 0.005, respectively) (times at which L1 and L2 induce pain related behavior, indicated with arrows in the figure, respectively). No such reduction in paw withdrawal latency was observed for PF (-Endo) except at 240 min.

Raw paw withdrawal response times (seconds) following injection with PF from women with or without endometriosis and the respective saline control over the course of 8 hours are provided in Supplementary Figure 2.3. Statistical analysis using LME models comparing treatments to saline (internal control) and with "time" as the interaction term, showed no interaction between time and treatment when comparing PF from women without endometriosis (-Endo) and saline, but a significant interaction (p: 0.0027) between time and PF (+) Endo versus saline was observed.

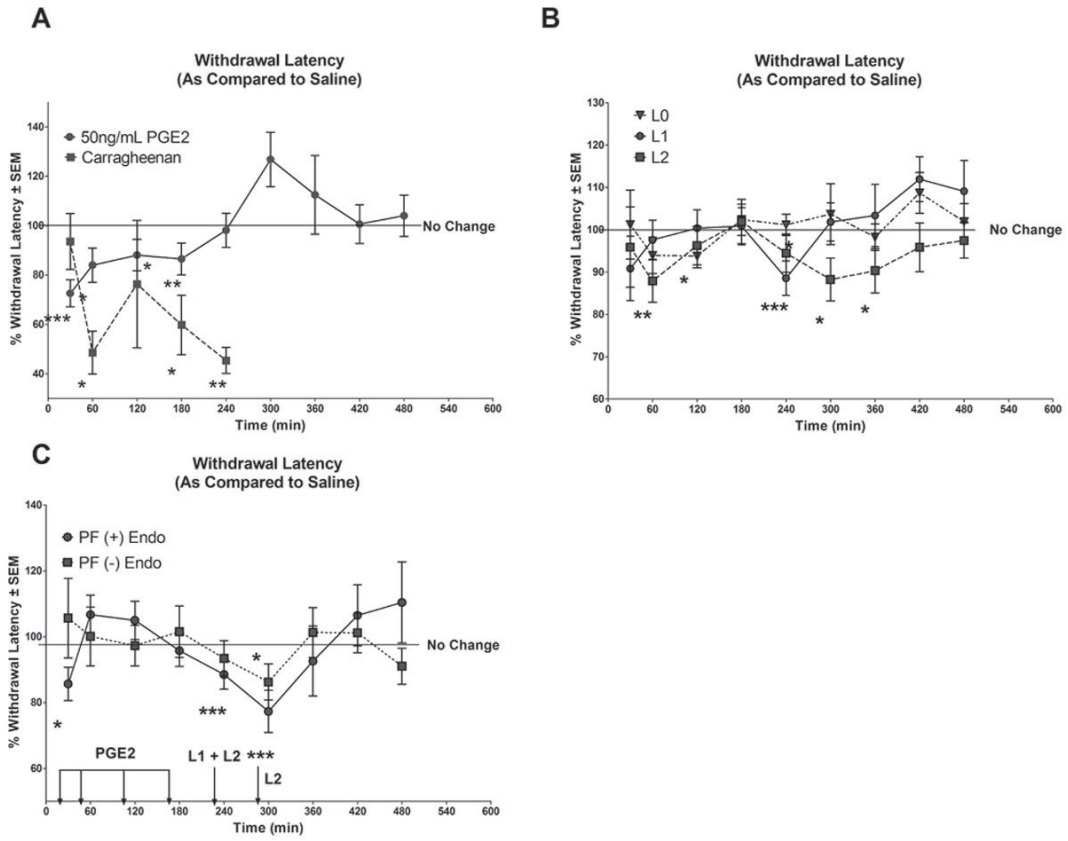


Figure 2.4. Oxidatively-modified LDLs induce nociception in Hargreaves assay. Paw withdrawal latency <100 in the Hargreaves assay indicates increased pain induced by the respective treatment. (A) Illustrates the % withdrawal latencies ± SEM (as compared to saline) following injection with known pain inducer carrageenan (3%) or PGE2 (50ng/mL). Significant reductions in withdrawal latency were observed at 60, 180 and 240 minutes (min) post-carrageenan injection. Significant reductions in withdrawal latency were observed post-PGE2 treatment at 30, 60, 120, and 180 minutes. (B) Illustrates the % withdrawal latencies ± SEM (as compared to saline) following injection with 100µg/ml L0, L1 or L2. Compared to saline, significant decreases in withdrawal latency were observed at 120min and 240min for L0 and L1 respectively and at 60, 240, 300 and 360min for L2. (C) Illustrates the % withdrawal latencies ± SEM (as compared to saline) following injection with 100µl PF (+) Endo or PF (-) Endo. Relative to saline, significant reductions in withdrawal latency were observed at 30, 240 min and 300 min following treatment with PF (+) Endo and at 240min following treatment with PF (-) Endo. Abbrev: PGE2: Prostaglandin E2; L0: Native LDL; L1: Minimally modified LDL; L2: oxidized LDL. PF (+) Endo: Peritoneal fluid from women with endometriosis; PF (-) Endo: Peritoneal fluid from control subjects; *: <0.05; **: <0.01; ***: <0.005. Arrows and labelled treatments above the arrow represent points of significant induction of pain related behavior by the stated treatment at the respective time points. The line presented at 100% indicates no change in response between treatment and saline. Statistical significance was determined using linear mixed effects models (LME) as described in the methods section.

Effect of Antioxidants (N-acetylcysteine or Vitamin E) and the NSAID Indomethacin on Induction of Pain Related Behavior by Oxidatively-modified LDL

We hypothesized that if non-enzymatic oxidation of LDL generated oxidation-sensitive factors of nociception, then oxidation of LDLs in the presence of N-acetylcysteine (1mM), vitamin E (50µM) or indomethacin (1µg) should decrease the generation of these nociceptive factors. LDLs oxidized in the presence of these agents were tested for their nociceptive ability using the Hargreaves assay. Figure 2.5A illustrates the % withdrawal latency ± SEM (as compared to saline) following injection with minimally-modified-LDL-L1 prepared in the presence of antioxidants or NSAID, i.e. L1+1mM N-acetylcysteine (n=23), L1+50µM vitamin E (n=34), and L1+1µg indomethacin (n=14). Compared to saline, treatment with L1+1mM N-acetylcysteine indicated a significant reduction in withdrawal latency at 60min and 360min post-injection (p: 0.005 and 0.045, respectively); however, no pain related behavior was observed at 240min post injection (the point in time where L1 alone induced a significant reduction in withdrawal latency (represented by arrow in the figure). Relative to saline, L1+50µM vitamin E indicated significant reductions in withdrawal latency at 120min, 180min and 240min post injection. There were no significant differences in withdrawal latency between L1 + 1µg indomethacin and saline. Figure 2.5B illustrates the % withdrawal latency ± SEM (as compared to saline) following injection with oxidized-LDL-L2 prepared in the presence of antioxidant or NSAID, i.e. L2+1mM N-acetylcysteine (n=18), L2+50µM vitamin E (n=19), and L2+1µg indomethacin (n=10). Compared to saline, L2+1mM N-acetylcysteine indicated significant reductions in withdrawal latency at 30min, 180min and 240min post injection (p: 0.040, 0.042 and 0.017, respectively). Compared to saline, treatment with either L2+50µM vitamin E or

L2+1µg indomethacin did not indicate any significant reductions in withdrawal latency, suggesting no pain related behavior response.

Raw paw withdrawal response times (seconds) for each treatment group and the respective saline controls over the course of 8 hours are provided in Supplementary Figure 2.4. Statistical analysis using LME models comparing treatments to saline (internal control) and with "time" as the interaction term, showed significant interaction (p: 0.037) between L1+1mM N-acetylcysteine and time versus saline but no such interactions with either L1+50µM vitamin E or L1+1µg indomethacin to saline.

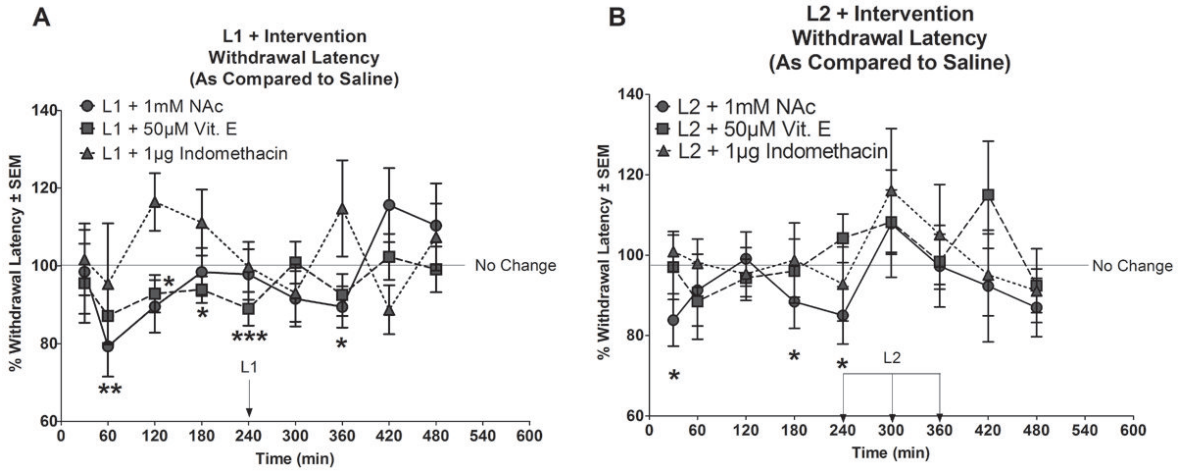


Figure 2.5. Withdrawal latencies of oxidatively-modified LDLs generated in the presence of N-acetylcysteine, Vitamin E or Indomethacin. (A) Illustrates the % withdrawal latencies ± SEM (as compared to saline) following injection with 100µg/mL L1 + 1mM NAc, L1 + 50µM Vit. E or L1 + 1µg indomethacin. Compared to saline, significant reductions in withdrawal latency were observed at 30 and 360min following treatment with L1 + 1mM NAc and 120, 180 and 240min following treatment with L1 + 50µM Vit. E. The reductions were lower compared to L1 alone (the time at which L1 alone induced pain related behavior-indicated as an arrow in the figure). No differences in withdrawal latency were observed between saline treatment and L1 + 1µg indomethacin. (B) illustrates the % withdrawal latencies ± SEM (as compared to saline) following injection with 100µg/mL L2 + 1mM NAc, L2 + 50µM Vit. E or L2 + 1µg indomethacin. Compared to saline, L2 + 1mM NAc indicated significant reductions in withdrawal latency at 30, 180 and 240 min post injection (arrows represent points of significant induction of pain related behavior by L2 alone). No differences in withdrawal latency was observed between saline and L2 + 50µM Vit E or L2 + 1µg indomethacin. *: <0.05; **:< 0.01; ***<0.005. The line presented at 100% indicates no change in response between treatment and saline. Statistical significance was determined using linear mixed effects models (LME) as described in the methods section.

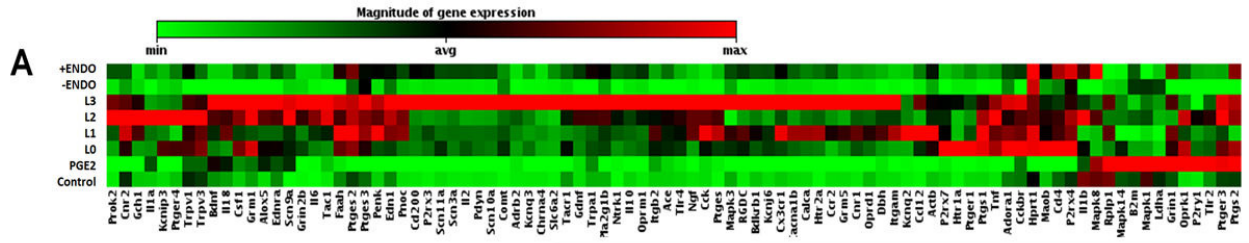
Human Cell Culture Studies

Oxidatively-modified LDLs Similar to PGE_2 Modulate Genes Involved in Nociception

A PCR array for neuropathic and inflammatory genes (Qiagen, Valencia, CA) was used to assess the similarities in regulation of genes involved in nociception by the oxidatively-modified lipoprotein preparations (L0-L3) and PGE_2. Ishikawa endometrial cells were either treated with LDL preparations or PGE_2. A list of differentially expressed genes obtained from the Human Pain: Neuropathic and Inflammatory Array is provided in the Supplemental Section (Supplementary Table 2.1). The clustergram in Figure 2.6A shows the relative expression of the 84 genes in the treatment groups compared to the Control group (cells treated with 1% charcoal-stripped media alone). Figure 2.6B shows a breakdown of the up- and down-regulation of genes within each treated group based on the fold change. Stringent cut-offs of 4-fold changes compared to the control group were used. In most cases, the expression of these genes increased with the increasing level of LDL oxidation (L3>L0), supporting the concept of increased nociception or inflammation with increasing non-enzymatic oxidation of LDLs. A table of notable genes altered due to various treatments is given in Figure 2.6C. Blue indicates a value that is < a 4-fold decreased expression compared to the control, while red values indicate a ≥4-fold increased expression from the control. Amongst the genes responsible for pain conduction, there was a distinct up-regulation in voltage-gated sodium channel (SCN10A, 11A, 3A and 9A) and opioid receptor (OPRD1 and M1) genes. About half of the genes associated with synaptic transmission (serotonin-HTR2A and glutamate receptors-GRIN2B and GRM5, calcium channels-CACNA1B) experienced similar up-regulation as a function of LDL oxidation level. The expression of key inflammatory genes such as interleukins-IL-2, IL-6, and fractalkine

receptor-1-CX3CR1, also increased in cells treated with increasing oxidation of LDL. PGE_2 treated cells compared to other treatments had a higher expression of PGE receptor 3 (PTGER3), the major receptors involved in PGE_2 mediated pain and fever.

The array was also used to study the changes in pain and inflammatory related genes in Ishikawa cells treated with PF obtained from women with (+Endo) and without (-Endo) endometriosis. Many genes that were differentially expressed by oxidatively-modified LDL treatments were similarly modulated in cells treated with PF from patients with endometriosis (Figure 2.6C) compared to control subjects.



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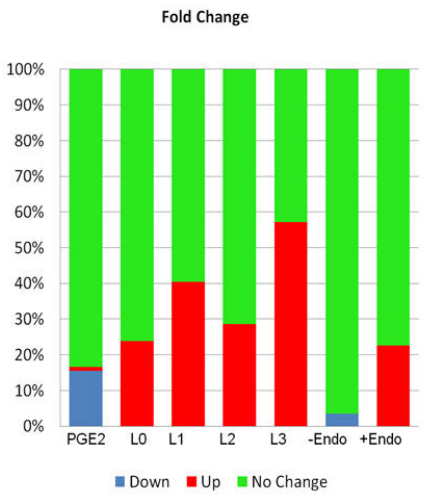

C

Gene ID	Description	PGE2	L1	- ENDO	+ENDO
CCL2	Chemokine (C-C motif) ligand 2	0.625	22.652	1.3299	6.0573
CCR2	Chemokine (C-C motif) receptor 2	0.1816	7.7486	0.6805	4.872
CX3CR1	Fractalkine receptor 1	0.1812	8.1204	0.7656	4.1926
IL2	Interleukin 2	0.3883	5.1789	1.0877	6.4026
IL6	Interleukin 6	1.3062	22.2539	1.7068	4.6439
NTRK1	Neurotrophic tyrosine kinase receptor type 1	1.0983	4.3619	0.9339	4.3681
OPRD1	Opioid receptor δ1	0.1173	4.2233	0.3647	1.3522
OPRM1	Opioid receptor μ1	0.3061	4.6139	1.238	6.7365
P2RX3	Purinergic receptor P2X, channel 3	0.601	6.1304	1.4587	7.9512
PLA2G1B	Phospholipase A2, group 1B	0.2708	2.2671	1.1418	2.8422
PNOC	Prepronociceptin	0.376	9.0588	1.1392	4.4061
PTGER3	PGE receptor 3	2.415	0.988	0.1461	0.541
PTGER4	PGE receptor 4	0.8697	0.9588	0.9758	1.1616
PTGES	PGE synthase	1.3305	20.3834	0.7378	3.451
PTGS1	Prostaglandin-endoperoxide synthase 1 (COX-1)	0.6607	6.1137	0.5749	1.5082
PTGS2	Prostaglandin-endoperoxide synthase 2 (COX-2)	1.6495	0.689	0.6035	1.4072
SCN10A	Sodium channel, type 10, α subunit	0.3047	5.1789	1.0877	6.4026
SCN11A	Sodium channel, type 11, α subunit	0.2363	3.8441	0.8074	4.7524
SCN3A	Sodium channel, type 3, α subunit	0.3047	5.1789	1.0877	6.4026
SCN9A	Sodium channel, type 9, α subunit	12.7168	12.4892	3.5259	8.2936

Figure 2.6. Human Pain-Nociceptive and inflammatory PCR array. Human Pain-Nociceptive and Inflammatory PCR array on Ishikawa endometrial cell lines that were treated with either PGE2 (50ng/ml) or the different forms of oxidatively modified LDLs (25μg/ml), or PF (100μl) from patients with endometriosis (+ Endo) or control women (-Endo) for 48 hours. (A) Clustergram of gene expression in cells treated with PGE2, oxidatively modified LDLs and peritoneal fluid. The array includes 84 inflammatory and nociceptive genes as well as housekeeping genes and quality control standards. (B) Represents the percent of genes that were classified as up-regulated, down-regulated, or no change compared to the control treatment based on a 4-fold cutoff of the fold change. (C) Represents notable genes based on fold change. Potential genes involved in the oxidatively-modified LDL mediated nociceptive pathway are highlighted with an arrow. While PGE2 and PF from women without endometriosis (-Endo) had very little change in gene expression, oxidatively-modified LDLs and endometriotic fluid from patients with endometriosis (+Endo) showed similar trends in induction of several nociceptive and inflammatory genes. [The supplementary Table 2.1 lists all the genes measured in the pain array]. Statistical analysis (T-test) was performed using the algorithm provided by the manufacturer (Qiagen).

DISCUSSION

The relationship between endometriosis and existence of pelvic pain symptoms is still not well understood. Hence, treatment still remains a major challenge. Prostaglandins synthesized by COX-1 and COX-2 are key mediators of pain and nociception, which are induced by cytokines and oxidative stress (Augoulea et al., 2012; Lousse et al., 2012). Lowering COX-2 levels is correlated to decreased endometriotic lesion size and number of implants (Komiyama, Aoki, Katsuki, & Nozawa, 2006; Machado et al., 2010). Peripheral inflammation increases pain sensitivity and COX-2 expression in the periphery and the central nervous system (Vardeh et al., 2009). Though NSAIDs are anti-inflammatory and anti-angiogenic in patients with endometriosis and animal models of endometriosis (Allen et al., 2009; Efstathiou et al., 2005) and used as the first line of treatment for pain, there is still no evidence that these agents are completely effective in relieving pain associated with endometriosis.

Oxidative stress, which is implicated in the etiology of several types of pain including chronic pelvic pain (Shahed & Shoskes, 2000), abdominal pain (Chi, Shiesh, & Lin, 2002) and fibromyalgia (Ozgocmen, Ozyurt, Sogut, & Akyol, 2006), induces NFkB and COX-2 and generates pro-inflammatory cytokines, TNF, IL-1β, NGF, nitric oxide (NO) and prostanoids. Oxidants such as superoxide (Z. Q. Wang et al., 2004) and NO donors (glyceryl trinitrite, sodium nitroprusside, SIN-1) can induce pain in humans (Aley et al., 1998; Petho & Reeh, 2012) and NOS inhibitors (N^G-methyl-L-arginine) can reduce inflammatory hyperalgesia in a PGE_2-dependent manner (Aley et al., 1998). 8-Isoprostanes are elevated in the prostatic fluid of patients with chronic pelvic pain and in the serum and plasma of patients with muscle injury (Shahed & Shoskes, 2000; Sinzinger, Lupattelli, Chehne, Oguogho, & Furberg, 2001). We and others have demonstrated the potential for an important role for oxidative stress (lipid peroxides)

in the etiology of endometriosis (Augoulea et al., 2012; Carvalho et al., 2012; Santanam, Song, Rong, Murphy, & Parthasarathy, 2002). In addition to cytokines, chemokines, and growth factors, there is an abundance of oxidative stress markers in the PF of women with endometriosis (Carvalho et al., 2012; Polak, Wertel, et al., 2013; Santanam, Murphy, et al., 2002). We and others have shown the presence of lipoproteins, especially LDL in the PF of women with endometriosis (Murphy, Santanam, Morales, et al., 1998; Murphy, Santanam, & Parthasarathy, 1998; Polak, Barczynski, et al., 2013; Turgut et al., 2013). Isolated LDL from the PF of women with endometriosis exhibited a higher electrophoretic mobility (oxidation increases the negative charge on the protein), lower levels of associated lipid antioxidants such as vitamin E and an increased *ex-vivo* oxidizability, thus suggesting that these lipoproteins are already in a slightly oxidized form (similar to the L1=minimally-modified LDL) (Murphy, Santanam, Morales, et al., 1998; Murphy, Santanam, & Parthasarathy, 1998). There is an abundance of transition metals such as copper and iron present in the PF of women with endometriosis that may contribute to the oxidation process (Defrere et al., 2011; Turgut et al., 2013). Our clinical study demonstrated that vitamin E supplementation not only reduced inflammatory and oxidative stress markers in the women with endometriosis, but also reduced their pain symptoms (Santanam et al., 2013), suggesting the possible role for oxidation sensitive nociception in these women.

Oxidatively-modified lipoproteins can cross-react with antibodies generated against prostaglandins, PGE_2 and PGF_2 (Proudfoot et al., 1995). This finding adds to the speculation that oxidatively-modified lipoproteins can mimic prostaglandin effects. This study demonstrated that non-enzymatic oxidatively modified lipoproteins (shown to be present in the PF of women of endometriosis) are similar to prostaglandins in their ability to modulate body temperature, induce nociception and alter the expression of inflammatory and nociceptive genes. LC-MS/MS studies

revealed that even non-enzymatic oxidation of LDLs generated both COX and LOX derived oxidation products of polyunsaturated lipids (PGE_2, PGD_2 and 11β-PGE_2 and 12, 15 and 5-HETEs). Hence, suppressing the generation of these molecules will probably necessitate the use of an antioxidant with or without conjunction with a COX inhibitor (NSAIDs).

It is known that the systemic inflammatory response to PGE_2 depends on its dose as well as its distribution. Centrally, as well as at low doses, PGE_2 causes fever, whereas peripherally and at higher doses, it elicits a hypothermic response (Morimoto, Long, Nakamori, & Murakami, 1991; Romanovsky, Simons, Szekely, & Kulchitsky, 1997). Non-prostaglandin eicosanoids such as seen in the oxidized LDL preparations can also play a role in thermoregulation (Kozak & Fraifeld, 2004). In our study, at the doses tested, i.c.v injection of LDL preparations resulted in hypothermia in contrast to the PGE_2 mediated hyperthermic reaction (body temperature). It has been shown that LPS as well as oxidants such as NO can cause hypothermia (Goteri et al., 2005; Koga et al., 2000; Monroy, Kuluz, He, Dietrich, & Schleien, 2001). In Hargreaves assay of nociception, we observed a time-sensitive pain related behavior by PGE_2 which peaked around 30 min and had no pain related behavior beyond 180min. Oxidized LDL preparations had varying pain related behavior responses which correlated with the extent of oxidation (L2>L1>L0). Interestingly, the oxidized LDL preparations had a dual pain related behavior response, with an initial early response (around 30-60 min), possibly due to the presence of PGE_2 followed by a later response (around 240-300 min) possibly due to the presence of other oxidized products. This dual response was similar to the pain related behavior response seen in the presence of PF from women with endometriosis (+Endo). These studies suggested both central and peripheral effects of oxidized LDL metabolites.

Antioxidants when given alone or in combination with analgesics lowered oxidative stress mediated pain (Cameron, Jack, & Cotter, 2001; Rokyta et al., 2003). Oxidized lipoproteins (L1-L2) generated in the presence of antioxidants N-acetylcysteine (aqueous antioxidant) and Vitamin E (lipid-soluble antioxidant) or the COX inhibitor (NSAID) indomethacin had no major effect on the initial pain related behavior responses (i.e. PGE2 mediated) but had a reduced capacity to induce pain related behavior at later time points (i.e. oxidized lipids mediated). The discrepancy in overall responses can be attributed to the dose of the agents tested as well as the extent to which these agents were able to inhibit the oxidation of LDL. Overall, our results support the notion that the use of antioxidants along with NSAIDs or other analgesics can reduce pain related behavior induced by oxidatively-modified LDL and may serve as potential therapeutic options for endometriosis-associated pain or other pain conditions. Though there were few instances where a null response or negative effect of dietary antioxidants (Kashanian, Lakeh, Ghasemi, & Noori, 2013; Trabert, Peters, De Roos, Scholes, & Holt, 2011) (Parazzini, Vigano, Candiani, & Fedele, 2013) or other agents such as raloxifene actually increasing chronic pelvic pain (Stratton et al., 2008) was observed, the majority of the studies with antioxidants had positive effects in ameliorating pelvic pain in endometriosis (Practice Committee of the American Society for Reproductive, 2014).

The Nociceptive and Inflammatory Pathway Gene Array revealed potential candidate genes that might be involved in nociception due to oxidatively-modified lipoproteins. We found that these lipoproteins do interact with similar receptors as PGE_2, but perhaps to a different extent. For example, there was very little change seen in the expression of PGE_2 receptors (e.g. PTGE3) when cells were treated with oxidatively-modified lipoproteins compared to PGE_2 but had higher induction of the opioid receptors, OPRD1 and OPRM1. DNA microarray analysis of

tissues obtained from patients with deep-endometriosis identified OPRM1 as one of the top three potential candidate genes involved in pain pathways (Matsuzaki, 2011; Matsuzaki et al., 2005; K. L. Ray, Mitchell, & Santanam, 2014) and both GnRH agonists and continuous oral progestin treatments reduced the expression of OPRM1 in these patients (Matsuzaki et al., 2007). Other genes that were differentially expressed in oxidized lipoprotein treated cells included voltage-gated sodium channels (SCN10A, 3A and 9A) (Dib-Hajj, Cummins, Black, & Waxman, 2010), which are known to be upregulated by COX derived prostanoids after inflammation (Gould et al., 2004), and the TRP family of ligand-gated ion channels (TRPV1 and TRPA1) (Patwardhan et al., 2010). Inflammatory genes (IL-6, IL-2, CX3CR1) were also activated, suggesting a role for these pathways in nociception. Interestingly, most of the genes that were modulated by oxidatively-modified lipoproteins were similar to those induced by cells treated with PF from women with endometriosis treated cells. The major limitations of our study include the use of non-endometriosis animal model to test pain responses, use of single doses of the tested agents and the use of a single nociceptive assay (Hargreaves). However, our findings are significant enough to support the presence of the oxidized lipid molecules in the PF of women with endometriosis and their potential role in endometriosis associated pain. The observation that oxidatively-modified lipoproteins are able to induce pain receptors and have the ability to modulate nociception makes them ideal candidates as therapeutic targets for pain in conditions such as the endometriosis-associated pain. Future studies using other animal models and in humans should explore the therapeutic use of targeting these agents in nociception.

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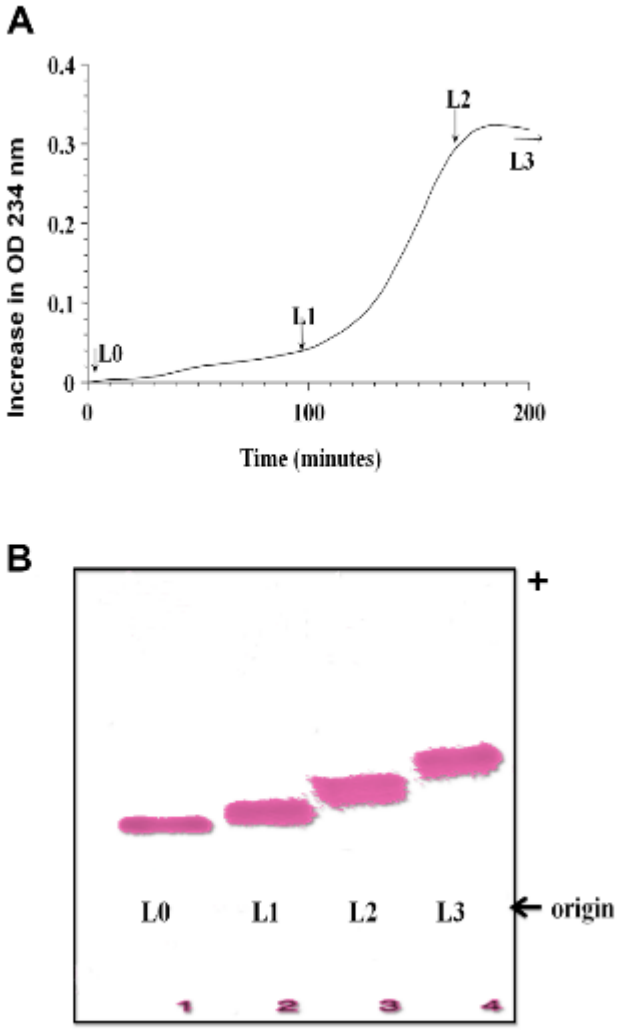
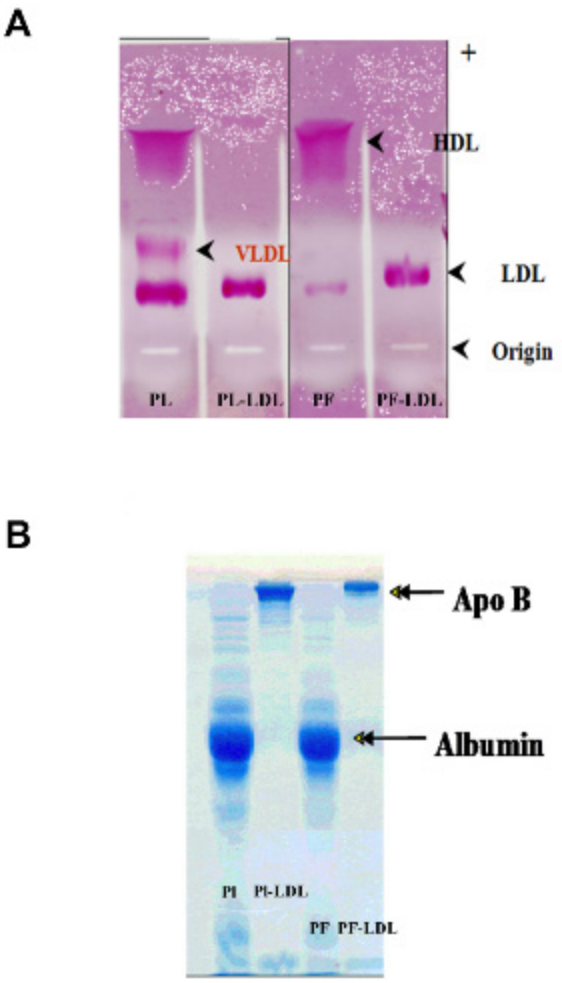
Author Disclosure Statement

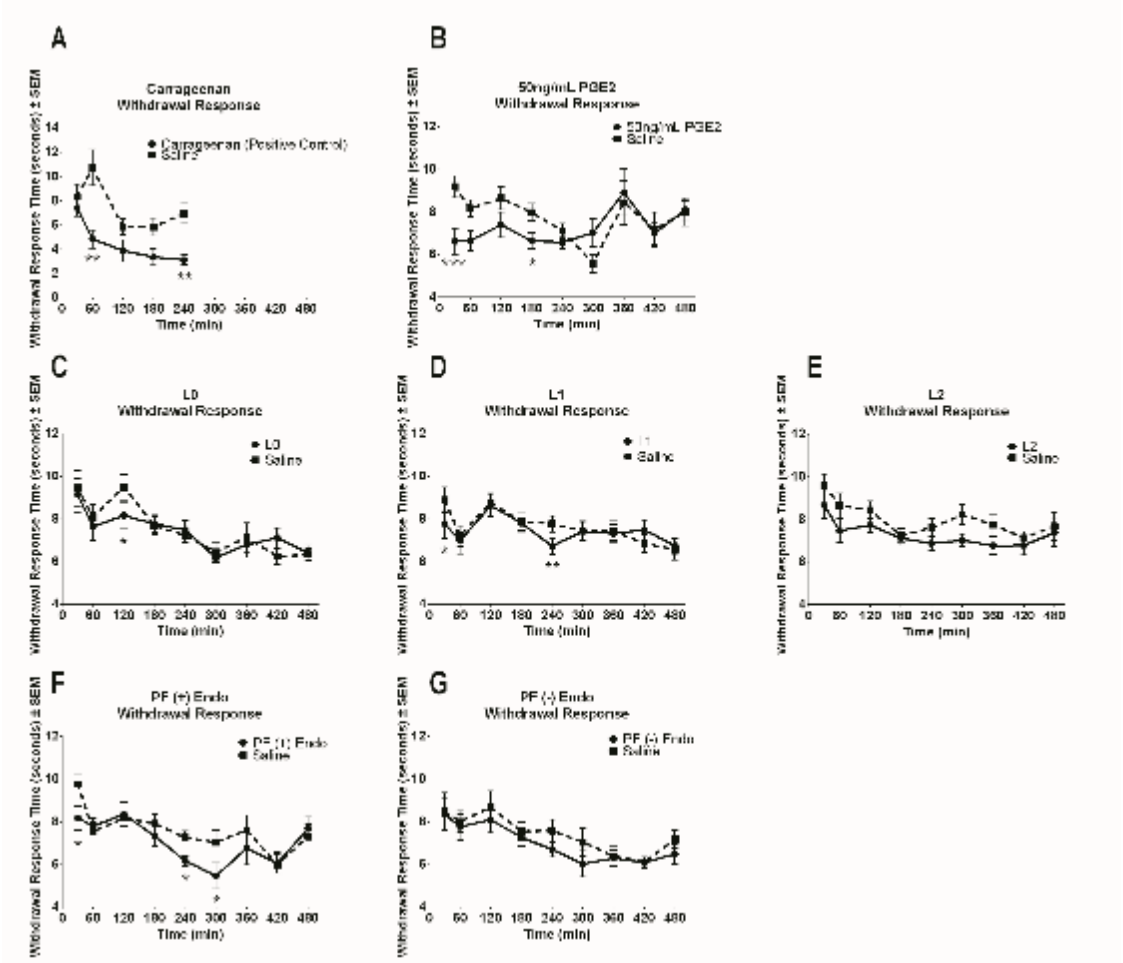
No competing financial interests exist. The authors indicate no conflicts of interest.



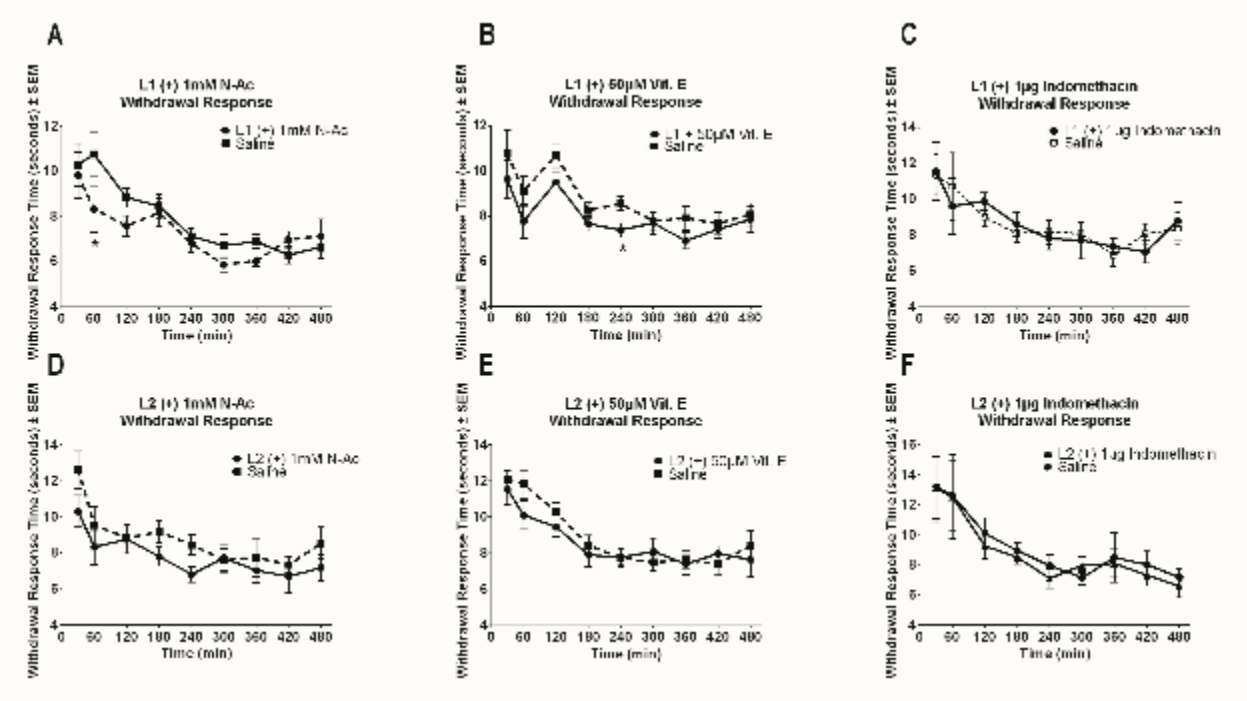
Supplementary Figure 2.1. Preparation of oxidatively modified LDL. (**A**) Lipoproteins undergo oxidation at different levels (Parthasarathy, Santanam, et al., 1998). The figure is a graphical representative of LDL that has undergone copper-mediated non-enzymatic oxidation. LDL preparations were collected at different time points during oxidation to represent, L0: native LDL at zero time, L1: minimally oxidized LDL, collected at the end of the lag-time, when the LDL begins to undergo oxidation, L2: oxidized LDL, collected at the end of LDL oxidation and L3: fully oxidized LDL, collected at the end of 24 hours of oxidation. (**B**) Representative figure of agarose gel electrophoresis showing increase in negative charge with increasing oxidation of LDL (L3>L2>L1>L0).



Supplementary Figure 2.2. Presence of lipoproteins in peritoneal fluid. (**A**) A representative figure of the agarose gel electrophoresis showing the presence of LDL, VLDL and HDL in the plasma, and the presence of LDL and HDL in peritoneal fluid. We have earlier published that while 90% of the PF samples from subjects with endometriosis were positive for the presence of lipoproteins, only 45% from normal subjects were positive for lipoproteins. We have shown earlier that the LDL isolated from PF was slightly more oxidized (increased negative charge) compared to plasma isolated LDL (Murphy, Santanam, Morales, et al., 1998; Murphy, Santanam, & Parthasarathy, 1998). **(B)** The image on the right is a representative native gel electrophoresis followed by Coomassie blue staining of lipoproteins. It shows the presence of intact apoB in the isolated LDL from PF.



Supplementary Figure 2.3. Raw paw withdrawal times (seconds). Paw withdrawal latencies (seconds) ± SEM across time (0-8hours) are shown. (**A)** Paw withdrawal latencies (seconds) ± SEM following injection of 3% carrageenan (right paw) and saline (left paw). Relative to saline, significant reductions in paw withdrawal latencies were observed at 60min, 180min and 240 min post carrageenan injection. (**B)** Paw withdrawal latencies (seconds) ± SEM following injection of 50ng/mL PGE_2 (right paw) and saline (left paw). Compared to saline, significant decreases in paw withdrawal latencies were observed at 30min, 60min, 120min and 180 min post PGE_2 injection. (**C-E)** Paw withdrawal latencies (seconds) ± SEM following injection of 100µL L0, L1 or L2 respectively (right paw) and saline (left paw). Compared to saline, a significant reduction in paw withdrawal latency was observed at 120min post L0 injection, at 240min post L1 injection and at 60min, 240min, 300min and 360min post L2 injection. (**F-G)** Paw withdrawal latencies (seconds) ± SEM following injection of 100µL PF from subjects with (+Endo) or without (-Endo) endometriosis (right paw) and saline (left paw). Compared to saline, significant decreases in paw withdrawal latencies were observed at 30min, 240min and 300min post PF (+Endo) injection and only around 240min post PF (-Endo) injection. Statistical significance was determined using linear mixed effects models (LME) as described in the method section. * p-value< 0.05; ** p-value< 0.01; *** p-value<0.005.



Supplementary Figure 2.4. Raw paw withdrawal times (seconds). Paw withdrawal latencies (seconds) ± SEM across time (0-8hours) are shown. **(A-C)** Paw withdrawal latencies (seconds) ± SEM following injection of 100µL L1 + 1mM NAc or 50µM Vit.E or 1µg Indomethacin (right paw) and saline (left paw). Compared to saline, L1 + 1mM NAc indicated significant reductions in paw withdrawal latencies at observed at 60min and 360min post injection and at 120 min, 180 min and 240min for 50µM Vit E post injection. No significant differences in withdrawal latencies were observed for L2 + 1µg Indomethacin post injection. **(D-F)** Paw withdrawal latencies (seconds) ± SEM following injection of 100µL L2 + 1mM NAc or 50µM Vit.E or 1µg Indomethacin (right paw) and saline (left paw). Compared to saline, L2 + 1mM NAc indicated significant reductions in withdrawal latency at 30 min, 180 min and 240 min post injection. No significant differences in withdrawal latencies were observed with either L2 +50µM Vit.E or L2 +1µg Indomethacin compared to saline. Statistical significance was determined using linear mixed effects models (LME) as described in the method section. * p-value< 0.05; *** p-value<0.005.

		Ishikawa cells treatment						
Gene ID	Description	PGE2	L0	L1	L2	L3	-ENDO	+ENDO
ACE	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	0.91	3.38	3.53	4.14	7.43	0.37	2.34
ADORA1	Adenosine A1 receptor	0.47	2.59	2.08	1.65	2.45	0.96	1.25
ADRB2	Adrenergic, beta-2-, receptor, surface	1.06	2.88	3.71	4.78	13.88	0.45	1.18
ALOX5	Arachidonate 5-lipoxygenase	2.21	2.49	1.77	3.03	4.06	0.80	1.35
BDKRB1	Bradykinin receptor B1	0.25	2.70	4.10	2.10	7.60	0.47	2.77
BDNF	Brain-derived neurotrophic factor	1.87	1.11	1.55	2.18	3.11	0.42	0.65
CACNA1B	Calcium channel, voltage-dependent, N type, alpha 1B subunit	0.25	3.41	8.59	2.19	10.49	0.81	3.48
CALCA	Calcitonin-related polypeptide alpha	0.26	3.30	9.09	3.29	11.18	0.90	2.95
CCK	Cholecystokinin	1.02	6.20	12.62	8.90	12.85	0.20	0.70
CCKBR	Cholecystokinin B receptor	0.98	2.65	2.02	1.88	2.57	0.38	1.28
CCL2	Chemokine (C-C motif) ligand 2	0.63	9.77	22.65	3.81	16.30	1.33	6.06
CCR2	Chemokine (C-C motif) receptor 2	0.18	1.95	7.75	3.06	13.95	0.68	4.87
CD200	CD200 molecule	0.47	3.34	3.68	3.15	12.24	0.96	6.31
CD4	CD4 molecule	0.15	2.98	1.93	1.86	1.25	0.84	2.51
CHRNA4	Cholinergic receptor, nicotinic, alpha 4	0.08	3.28	4.28	2.76	19.80	0.32	1.19
CNR1	Cannabinoid receptor 1 (brain)	0.28	6.38	12.72	5.96	18.33	0.84	3.80
CNR2	Cannabinoid receptor 2 (macrophage)	0.31	1.25	1.80	1.96	1.42	0.61	0.86
COMT	Catechol-O-methyltransferase	0.98	1.64	1.53	1.43	2.60	1.37	1.32
CSF1	Colony stimulating factor 1 (macrophage)	1.35	2.72	1.76	2.80	3.43	0.75	1.47
CX3CR1	Chemokine (C-X3-C motif) receptor 1	0.18	3.19	8.12	3.00	8.35	0.77	4.19
DBH	Dopamine beta-hydroxylase (dopamine beta-monooxygenase)	0.83	1.98	5.22	3.26	7.78	0.51	1.51
EDN1	Endothelin 1	0.45	1.34	2.24	2.51	2.85	1.09	1.60
EDNRA	Endothelin receptor type A	1.21	1.55	1.14	1.78	2.43	0.59	1.24
FAAH	Fatty acid amide hydrolase	0.82	1.45	1.73	1.39	1.66	0.79	1.32
GCH1	GTP cyclohydrolase 1	0.82	1.15	1.95	2.58	1.81	0.95	0.92
GDNF	Glial cell derived neurotrophic factor	0.97	1.94	2.06	4.06	6.16	0.66	2.81
GRIN1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1	2.15	1.43	1.65	0.71	1.53	0.42	1.69
GRIN2B	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B	0.92	3.36	2.55	4.92	7.55	1.42	1.34
GRM1	Glutamate receptor, metabotropic 1	1.25	3.05	1.42	3.23	3.26	0.51	1.37
GRM5	Glutamate receptor, metabotropic 5	0.12	2.86	8.20	2.25	13.44	0.36	2.27
HTR1A	5-hydroxytryptamine (serotonin) receptor 1A	0.33	34.05	4.56	4.21	16.71	1.56	5.63
HTR2A	5-hydroxytryptamine (serotonin) receptor 2A	0.83	5.08	14.31	5.77	17.06	1.44	5.40
IL10	Interleukin 10	0.23	2.74	3.10	3.79	9.78	0.66	3.64
IL18	Interleukin 18 (interferon-gamma-inducing factor)	1.51	1.05	1.99	1.84	2.56	0.66	0.82
IL1A	Interleukin 1, alpha	1.81	1.37	1.59	3.97	1.27	0.68	1.36
IL1B	Interleukin 1, beta	0.64	0.78	0.43	1.23	0.69	0.35	0.92
IL2	Interleukin 2	0.39	6.03	5.18	3.20	17.23	1.09	6.40
IL6	Interleukin 6 (interferon, beta 2)	1.31	13.96	22.25	40.95	53.92	1.71	4.64
ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	0.14	2.37	11.24	5.19	13.45	0.35	1.32
ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	1.30	2.39	3.16	2.49	4.53	0.64	1.28
KCNIP3	Kv channel interacting protein 3, calsenilin	0.90	1.83	1.24	2.34	1.21	1.01	1.09
KCNJ6	Potassium inwardly-rectifying channel, subfamily J, member 6	0.35	4.24	4.47	2.02	10.34	0.65	3.84
KCNQ2	Potassium voltage-gated channel, KQT-like subfamily, member 2	0.55	3.63	20.53	5.02	5.32	0.35	2.41
KCNQ3	Potassium voltage-gated channel, KQT-like subfamily, member 3	0.58	3.02	2.30	2.78	9.52	0.66	2.25
MAOB	Monoamine oxidase B	0.90	1.79	1.36	1.40	1.29	1.06	1.35
MAPK1	Mitogen-activated protein kinase 1	1.29	0.61	0.52	0.76	0.69	0.80	0.46
MAPK14	Mitogen-activated protein kinase 14	1.70	1.11	0.64	0.92	0.90	0.75	0.66
MAPK3	Mitogen-activated protein kinase 3	0.73	1.43	1.75	0.78	2.44	1.02	1.33

MAPK8	Mitogen-activated protein kinase 8	1.07	0.64	0.64	0.98	0.73	0.57	1.19
NGF	Nerve growth factor (beta polypeptide)	0.21	3.01	10.73	10.67	15.39	0.52	1.59
NTRK1	Neurotrophic tyrosine kinase, receptor, type 1	1.10	3.17	4.36	5.88	11.55	0.93	4.37
OPRD1	Opioid receptor, delta 1	0.12	2.19	4.22	2.27	7.33	0.36	1.35
OPRK1	Opioid receptor, kappa 1	2.39	2.19	1.45	2.23	1.24	0.92	1.39
OPRM1	Opioid receptor, mu 1	0.31	4.98	4.61	7.63	16.37	1.24	6.74
P2RX3	Purinergic receptor P2X, ligand-gated ion channel, 3	0.60	5.01	6.13	3.58	15.86	1.46	7.95
P2RX4	Purinergic receptor P2X, ligand-gated ion channel, 4	0.63	1.55	1.32	1.40	1.16	1.12	1.49
P2RX7	Purinergic receptor P2X, ligand-gated ion channel, 7	0.70	6.74	3.58	3.10	3.58	0.46	1.14
P2RY1	Purinergic receptor P2Y, G-protein coupled, 1	2.89	1.49	1.31	1.94	0.93	0.93	1.26
PDYN	Prodynorphin	0.38	3.91	3.92	2.60	13.06	0.82	4.85
PENK	Proenkephalin	0.34	2.94	7.79	5.41	6.80	0.76	4.05
PLA2G1B	Phospholipase A2, group IB (pancreas)	0.27	2.14	2.27	3.53	5.36	1.14	2.84
PNOC	Prepronociceptin	0.38	3.39	9.06	8.58	11.57	1.14	4.41
PROK2	Prokineticin 2	0.72	2.16	3.51	9.13	6.81	1.14	3.50
PTGER1	Prostaglandin E receptor 1 (subtype EP1), 42kDa	0.14	12.22	4.26	2.62	4.59	0.61	2.81
PTGER3	Prostaglandin E receptor 3 (subtype EP3)	2.42	1.78	0.99	2.47	2.33	0.15	0.54
PTGER4	Prostaglandin E receptor 4 (subtype EP4)	0.87	1.58	0.96	2.00	1.15	0.98	1.16
PTGES	Prostaglandin E synthase	1.33	9.40	20.38	15.04	23.22	0.74	3.45
PTGES2	Prostaglandin E synthase 2	0.36	1.70	2.09	1.84	1.89	0.87	1.62
PTGES3	Prostaglandin E synthase 3 (cytosolic)	0.26	1.73	2.36	2.22	2.89	1.57	1.53
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	0.66	5.88	6.11	5.17	3.84	0.57	1.51
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.65	1.46	0.69	1.44	1.49	0.60	1.41
SCN10A	Sodium channel, voltage-gated, type X, alpha subunit	0.30	4.70	5.18	3.36	17.23	1.09	6.40
SCN11A	Sodium channel, voltage-gated, type XI, alpha subunit	0.24	5.59	3.84	3.02	13.13	0.81	4.75
SCN3A	Sodium channel, voltage-gated, type III, alpha subunit	0.30	4.70	5.18	4.64	17.23	1.09	6.40
SCN9A	Sodium channel, voltage-gated, type IX, alpha subunit	12.72	13.40	12.49	29.21	27.94	3.53	8.29
SLC6A2	Solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	0.30	4.70	5.18	3.20	29.27	1.09	6.40
TAC1	Tachykinin, precursor 1	1.92	3.30	6.06	12.49	13.04	0.55	2.28
TACR1	Tachykinin receptor 1	0.32	1.94	1.04	1.83	3.69	0.50	1.36
TLR2	Toll-like receptor 2	2.63	1.21	1.10	1.76	1.21	0.56	0.89
TLR4	Toll-like receptor 4	0.75	3.23	3.77	4.50	7.42	0.47	1.50
TNF	Tumor necrosis factor	0.50	9.11	7.83	10.89	8.74	0.54	2.54
TRPA1	Transient receptor potential cation channel, subfamily A, member 1	1.32	1.86	1.68	3.04	4.65	0.37	2.66
TRPV1	Transient receptor potential cation channel, subfamily V, member 1	0.81	1.34	1.25	1.90	1.33	0.34	1.13
TRPV3	Transient receptor potential cation channel, subfamily V, member 3	0.81	1.75	1.63	2.27	1.43	0.19	0.77

Supplementary Table 2.1. Differentially expressed genes from Human Pain: Neuropathic and Inflammatory Array. Expression of genes involved in nociception and inflammation **Red** values indicate 4-fold up-regulation compared to control; **blue** values indicate 4-fold down-regulation compared to control. Expression often increased with level of LDL oxidation, with L3 treated cells typically having the highest expression. Cells treated with peritoneal fluid from endometriosis patients (+ENDO) had higher expression compared to control (-ENDO) peritoneal fluid samples, providing molecular evidence for increased pain and inflammation associated with endometriosis. Additionally, levels of PGE_2 receptors and COX-2 were comparable between the PGE_2 group and oxidatively-modified lipoprotein treated cells.

CHAPTER 3

REDOX REGULATION OF MICRORNAS IN ENDOMETRIOSIS-ASSOCIATED PAIN

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ABSTRACT

Endometriosis is a chronic, painful condition with unknown etiology. A differential expression of microRNAs (miRNAs) in the endometriotic tissues from women with endometriosis with pain compared to those without suggested a plausible role for miRNA or epigenetic mechanisms in the etiology of endometriotic pain. The peritoneal milieu is involved in maintenance of endometriotic lesion and nociception. We recently showed the mechanistic role for oxidized-lipoproteins (ox-LDLs) present in peritoneal fluid (PF) in endometriosis and pain. We explored the possibility of ox-LDL modulating the expression of miRNAs in a manner similar to PF from women with endometriosis. Expression levels of miRNAs and their predicted nociceptive and inflammatory mRNA targets were determined in PF and ox-LDL-treated human endometrial cell lines. Samples from IRB-approved and consented patients with and without endometriosis or pain were used. These were compared to endometrial cell lines treated with various forms of oxidized-lipoproteins. RNA (including miRNAs) were isolated from treated endometrial cells and expression levels were determined using commercial miRNome arrays. Cell lysates were used in immunoblotting for inflammatory proteins using a protein array. Twenty miRNAs

including isoforms of miR-29, miR-181 and let-7 were mutually differentially expressed in cells treated with PF from endometriosis patients with pain and those treated with ox-LDL components. The ox-LDL and endo-PF treatment also produced significant overexpression of microRNA predicted target genes *nerve growth factor*, *interleukin-6* and *prostaglandin E synthase* and overexpression of their downstream protein targets Mip1a and MCP1. This study showed similarities between miRNA regulation in PF from endometriotic women and ox-LDLs present in abundance in the PF of these women. Key miRNAs responsible for targeting nociceptive and inflammatory molecules were downregulated in the presence of ox-LDLs and endo-PF, thus playing a role in the etiology of endometriotic pain. These redox-sensitive miRNAs can be of potential use as targets in the treatment of endometriosis-associated pain.

INTRODUCTION

Endometriosis is a gynecological disorder that affects 5-15% of women of childbearing age and 3-5% of post-menopausal women worldwide (Giudice, 2010; Vigano, Parazzini, Somigliana, & Vercellini, 2004). It is defined by the presence of endometrial cells implanted in an extra-uterine location and can be asymptomatic or present with a wide range of symptoms, including infertility and a number of chronic pelvic pain conditions (Greene et al., 2016; Taylor et al., 2012). Despite the intensity of some of these symptoms, endometriosis often goes undiagnosed for several years (Hadfield, Mardon, Barlow, & Kennedy, 1996; Kavoussi, Lim, Skinner, Lebovic, & As-Sanie, 2016).

Numerous mechanisms of endometriosis-associated pain and inflammation have been proposed over the years (Laux-Biehlmann, d'Hooghe, & Zollner, 2015; B. D. McKinnon, Bertschi, Bersinger, & Mueller, 2015). It is also known that endometriosis is a hormonal disorder, and heightened levels of estrogen are associated with increased inflammation and

nociception (Liang & Yao, 2016; Zhao et al., 2015). Prostaglandin E2 is an example of an overexpressed inflammatory nociceptive molecule involved in pain associated with endometriosis which can have further downstream effects (Arosh et al., 2015; Y. Liu et al., 2011). It is also believed that endometriotic lesions release chemotactic molecules such as monocyte chemotactic protein-1 (MCP-1) and fractalkine (CX3CL1) that attract immune cells into the peritoneal cavity (Ahn et al., 2015; Y. Wang et al., 2014). These cells trigger the secretion of more cytokines and growth factors such as IL-6, IL-8, and TNF-α, further promoting lesion growth (X. Cao, Yang, Song, Murphy, & Parthasarathy, 2004; Hou, Zhou, Wang, & Li, 2016; Na et al., 2011; Rong et al., 2002; Santanam et al., 2013). All of these molecules accumulate in the peritoneal fluid (PF), creating a dynamic milieu of inflammatory and nociceptive mediators which plays a role in the etiology of endometriosis (Bedaiwy & Falcone, 2003; Kyama et al., 2009; Mahnert, Morgan, Campbell, Johnston, & As-Sanie, 2015; B. D. McKinnon et al., 2015).

Over the years, our laboratory has provided evidence for the role of oxidative stress in the etiology of endometriosis and its associated pain (Murphy, Santanam, & Parthasarathy, 1998; Santanam et al., 2013; Santanam, Murphy, et al., 2002; Santanam, Song, et al., 2002). We showed increased presence of oxidatively modified proteins in the PF and endometrium/endometriotic tissue (Murphy, Santanam, Morales, et al., 1998; Shanti, Santanam, Morales, Parthasarathy, & Murphy, 1999). Oxidatively modified LDLs present in the PF increased the proliferation of endometrial cells and the expression of MCP-1 (Rong et al., 2002). We recently showed the nociceptive role for oxidatively modified low-density lipoproteins (ox-LDLs) in endometriosis-associated pain (K. Ray et al., 2015) and the ability of antioxidant supplementation to lower inflammation and chronic pelvic pain in women with endometriosis

(K. Ray et al., 2015; Santanam et al., 2013; Santanam, Zoneraich, & Parthasarathy, 2016). Though many nociceptive molecules including ox-LDLs have been identified, the mechanism through which these molecules promote endometriosis-associated pain is still unclear.

The etiological role of epigenetics in health and disease is ever-expanding. This concept of mRNA alterations without changes to the gene sequences has become part of the paradigm in studying many disease conditions in humans (Bird, 2007; Calicchio, Doridot, Miralles, Mehats, & Vaiman, 2014). Often included as a regulator in epigenetics are microRNAs (miRNAs), short RNAs (about 23 nucleotides) which are capable of regulating gene expression at the transcriptional, post-transcriptional, and translational levels by binding to complementary sequences on target mRNA (Andersen et al., 2014; Bartel, 2009). It has long been stated that miRNA regulation occurs in one of two ways: i) the target mRNA is degraded when a miRNA seed sequence perfectly complements with the target mRNA sequence, or ii) translation is impaired when there is imperfect matching between the miRNA-mRNA sequences, leading to gene silencing (Deng et al., 2008; Mari-Alexandre et al., 2016). However, recent discoveries provide evidence that miRNAs in eukaryotes, zebrafish, and Drosophila predominantly repress translation of new mRNA targets, succeeded by deadenylation and degradation of the targets (Burney & Giudice, 2012; K. L. Ray et al., 2014). Interestingly, gene activation by miRNAs is also plausible. This can occur directly via targeting of the mRNA by miRNA, or indirectly by repressing nonsense-mediated RNA decay (Vasudevan et al., 2007).

MiRNAs have a crucial role in cellular homeostasis, which explains why alterations in their expression or function have been associated with diseased states including certain cancers (Jin et al., 2014; X. Y. Li et al., 2014; C. Liu et al., 2016), neurodegenerative disorders (Kumar et al., 2013; Majidinia et al., 2016; Miller et al., 2012), and cardiovascular and respiratory

conditions (JF, Neylon, McGorrian, & Blake, 2016; X. Wang, Du, & Li, 2013; Zhou et al., 2016). Fluid-based miRNA (serum, saliva, sputum, cerebrospinal fluid, plasma, whole blood, and urine) profiling could provide invaluable information for studies where the disease is not derived from only one type of cell or a specific type of cell. This possibility has opened doors for non-invasive diagnostic techniques in various disease states (Bhomia, Balakathiresan, Wang, Papa, & Maheshwari, 2016; Igaz & Igaz, 2015; Leidinger et al., 2011; Rajasekaran, Pattarayan, Rajaguru, Sudhakar Gandhi, & Thimmulappa, 2016; Umemura & Kuroki, 2015). Hence miRNAs are considered good therapeutic targets in cancer and cardiovascular disease (C. Li, Feng, Coukos, & Zhang, 2009; Mishra, Tyagi, Kumar, & Tyagi, 2009; L. M. Tsai & Yu, 2010).

Very few studies have explored the possible association between miRNA-mediated regulation and reproductive diseases such as endometriosis. Recent studies have speculated that endometriosis is an epigenetic disease (Borghese, Zondervan, Abrao, Chapron, & Vaiman, 2016; S. W. Guo, 2009; Izawa, Taniguchi, Terakawa, & Harada, 2013). MiRNAs play a major role in the development of endometriotic lesions by contributing to mechanisms involving hypoxic injury, inflammation, tissue repair, cell proliferation, extracellular matrix remodeling, and angiogenesis (Mari-Alexandre et al., 2015; Ohlsson Teague et al., 2009). In endometriosis, miRNA profiling studies have compared ectopic versus eutopic endometrial tissues (Filigheddu et al., 2010; Ohlsson Teague et al., 2009; Pan & Chegini, 2008), often concluding that many miRNAs are differentially expressed between the two groups and target genes closely associated with endometriosis. Studies investigating miRNA profiles in eutopic tissues from women with and without endometriosis (Burney et al., 2009; Hawkins et al., 2011; Laudanski et al., 2013) showed a trend of downregulated miRNA levels in tissues from women with endometriosis. Wang and colleagues also showed global downregulation in the circulating levels of miRNAs in

the serum of women with endometriosis, with 91% of significantly differentiated miRNAs showing decreased expression in endometriosis patients (L. Wang et al., 2016). There are very few studies that have measured miRNAs in the peritoneal fluid (PF), which is the most dynamic component and major player in the etiology of endometriosis (Castro et al., 2010; Y. Liu et al., 2011; Loh et al., 1999).

With our continued interest in understanding the etiology of the pain associated with endometriosis, we profiled miRNAs in endometriotic tissues obtained from women with endometriosis and pain and compared it to eutopic tissue from women without endometriosis. Since we recently identified that ox-LDLs parallel nociceptive responses similar to PF from women with endometriosis-associated pain (K. Ray et al., 2015), we hypothesized that these lipoprotein components function through modulating miRNAs that regulate inflammatory and nociceptive genes in endometriosis. We compared the miRNA profile of PF treated endometriotic cells to Ox-LDL treated cells. We validated miRNA regulation by assessing the levels of their predicted target genes. Our results identified miRNAs that play a role in endometriosis-associated pain. Targeting these redox-sensitive miRNAs may be a novel approach to treat endometriosis-associated pain.

MATERIALS AND METHODS

Human Subject Participants

Women ages 21 to 60 years undergoing tubal ligation or having non-endometriosis disorders (controls) or patients with endometriosis- endo (laparoscopically diagnosed or patients with symptoms followed by pathological confirmation) were recruited from the Obstetrics-Gynecology clinic at Cabell Huntington Hospital, Joan C Edwards School of Medicine, Marshall University, in Huntington, WV. This HIPAA compliant study was approved by the Institutional

Review Board of the Marshall University School of Medicine and was carried out according to the principles of the Declaration of Helsinki. All patients were consented prior to the study. All women completed a gynecologic/infertility history form, a pre-operative quality of life questionnaire and assessment of pain using a visual analog scale for assessment of endometriosis associated pain (dysmenorrhea, non-menstrual pelvic pain, dyspareunia, and dyschesia) (adapted from the validated International Pelvic Pain Society's Pelvic Assessment Form). Date of their last menstrual period was used to assess their cycle time. The inclusion criteria included women ages 21-60 years old, with normal menstrual cycles and otherwise in normal health (except for pain and endometriosis) who have not been on any hormonal medication for at least one month before sample collection. Exclusion criteria included subjects with current medical illnesses such as diabetes, cardiovascular disease, hyperlipedemia, hypertension, systemic lupus erythematosis or rheumatologic disease, positive HIV/AIDS, active infection. Subjects were asked to stop multivitamins that contain high levels of antioxidants and anti-inflammatory medications one month prior to sample collection.

Peritoneal Fluid Collection

Peritoneal fluid (PF) (devoid of blood contamination) was collected on ice from all women during laparoscopic surgery. Peritoneal fluid was spun at 2000xg to remove any cellular debris. The supernatant was used immediately for studies or stored in a -80^0C freezer for future use.

Endometrial Tissue Collection and RNA Isolation

Endometrial (eutopic) tissues from control patients and ectopic endometriotic tissues from endometriosis (ovarian or peritoneal endometriosis) patients were removed during laparoscopy/laparotomy by a qualified physician. Biopsy fragments were immediately placed in

RNA*later* solution (Qiagen, Gaithersburg, MD) and subsequently stored in a freezer at -80°C. RNA extraction from 100 mg of tissue (eutopic and ectopic) was carried out using Qiazol Lysis Reagent (Qiagen). Tissues were homogenized using zirconium oxide beads in a Bullet Blender® homogenizer (Next Advance, USA) and RNA was isolated using the Qiagen miRNeasy Mini Kit following the manufacturer's recommendations. The quantity and quality of RNA were measured in the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Cell Culture and RNA Isolation

Ishikawa cells, a human (39-year-old woman) established endometrial cell line (Sigma-Aldrich, St. Louis, MO), were cultured in T75 flasks in complete media (DMEM/F12, 10% FBS, 1% Pen/Strep, 1% L-glutamine). These cells were used because they express characteristics similar to those of mature endometrial epithelial cells (Bulun et al., 2006; Cho, Mutlu, Zhou, & Taylor, 2016; Nishida, Kasahara, Kaneko, Iwasaki, & Hayashi, 1985). Approximately 70% confluent cells were treated with either 25 µg of various LDL preparations (ox-LDLs, as described previously (K. Ray et al., 2015)) or 1% PF from patients for 48 hours in a DMEM/F12 media containing 1% charcoal-stripped FBS. Briefly, LDL isolated from plasma (human volunteers) was oxidized using copper. Extent of oxidation was determined by the formation of conjugated diene at OD 234 nm. The oxidation process was terminated at specific time points to generate various forms of ox-LDL preparations: (a) native LDL (L0), (b) minimally-modified LDL (L1, usually terminated at the end of the lag time), (c) oxidized LDL (L2, after the oxidation has reached its plateau) and (d) completely or fully oxidized LDL (L3, after 24 hours of oxidation) (Parthasarathy, Auge, et al., 1998; Parthasarathy et al., 2010; Parthasarathy, Santanam, et al., 1998; K. Ray et al., 2015). Patient peritoneal fluid (PF) groups were +endo/+pain (YY), +endo/-pain (YN), and –endo/-pain (NN, "control fluid"). The concentrations

chosen were selected from our previous published studies (K. Ray et al., 2015). At the end of 48 hours, cells were collected using Qiazol reagent and RNA was isolated using the Qiagen miRNeasy Mini Kit. The quantity and quality of RNA were measured in the NanoDrop 2000 spectrophotometer. Cells were also collected in RIPA buffer containing protease inhibitors and protein concentrations were measured using a modified Lowry protocol.

RT^2 MiRNome Array

Total RNA (which includes miRNA) isolated from the tissues and treated cells using MiRNeasy kit (Qiagen) were used. cDNA synthesis from 2 µg of each sample was performed using miScript II RT Kit (Qiagen). MiRNA expression was analyzed in the cDNA samples using the commercial Human miRNome PCR Array (MIHS-3216Z; Qiagen) on the Roche LightCycler 480 system (Roche, Indianapolis, IN). Fold change was determined using Pfaffl equation (2^{-ddCt}) for all groups compared with eutopic tissue from control women (tissues) or media control (cells) using the manufacturer's algorithm, which uses a t-test as the default statistics to compare differences using five SNORDs and RNU6 as housekeeping genes. A *p*-value less than 0.05 was used to identify significantly differentially expressed miRNAs in treated Ishikawa cells or in endometriotic tissues.

Real-Time PCR Analysis for Gene Expression

cDNA synthesis from 1 µg of RNA isolated from each cell treatment was prepared using iScript cDNA Synthesis Kit (Biorad, Hercules, CA). Expression of nerve growth factor (*NGF*); interleukin-6 (*IL-6*); cannabinoid receptor 1 (*CNR1*); Sodium Channel, Voltage Gated, Type XI Alpha Subunit (*SCN11A*); and prostaglandin E synthase 3 (*PTGES3*) in cells were analyzed using the Applied Biosystems OneStepPlus Real-Time PCR system (Thermo Scientific). Primers used in the experiment are listed in Supplementary Table 3.1. Fold change was determined using

Pfaffl equation (2^{-ddCt}) for all groups compared with 1% charcoal-stripped serum media alone. A *p*-value less than 0.05 was used to identify significantly differentially expressed mRNAs in Ishikawa cells treated with PF and ox-LDLs compared with the charcoal-stripped media treated cells (control group).

Immunoblotting

Cell lysates were prepared from PF or ox-LDL-treated Ishikawa cells using RIPA buffer containing protease inhibitors. The Human Neuro Discovery Array C1 (RayBiotech, Inc., Norcross, GA), which includes 20 human neurologically relevant proteins belonging to immune response and inflammation pathways was used for the detection of changes in target proteins. This array was chosen because it includes several proteins that play a role in neuronal and peripheral nociception and inflammation. The manufacturer's suggested protocol for analysis was followed. In brief, the provided membranes were blocked for 30 minutes prior to sample treatment and then incubated with samples overnight at 4°C. Following washing, the membranes were then incubated with a biotinylated detection antibody cocktail overnight (4°C), washed, and incubated with horseradish peroxidase (HRP)-conjugated streptavidin. Following additional washing steps, the membrane was incubated in the detection buffer followed by imaging of the developed proteins using the ChemiDoc system (Biorad). Results were analyzed using the manufacturer's Analysis Tool Excel-based software (RayBiotech, Inc.).

Targetscan and Ingenuity Pathway Analysis

TargetScan Human 7.0 online database (www.targetscan.org) was used to identify miRNA target genes. The list of differentially expressed miRNAs in PF and ox-LDL-treated cells was uploaded into Ingenuity Pathway Analysis (IPA, Qiagen), along with the cytokines

analyzed using the protein array. IPA was used to identify any relationship among the differentially expressed miRNAs and cytokines, either via direct or indirect interactions.

Statistical Analysis

Prism software (GraphPad, Inc., La Jolla, CA) was used for analysis of non-array qPCR data in human tissue and cell culture studies. All values were expressed as mean ± standard error of the mean (SEM). One-way ANOVA followed by Tukey's post-hoc test was used to detect differences in relative gene expression among treatment groups. *P* values less than 0.05 were considered significant.

RESULTS

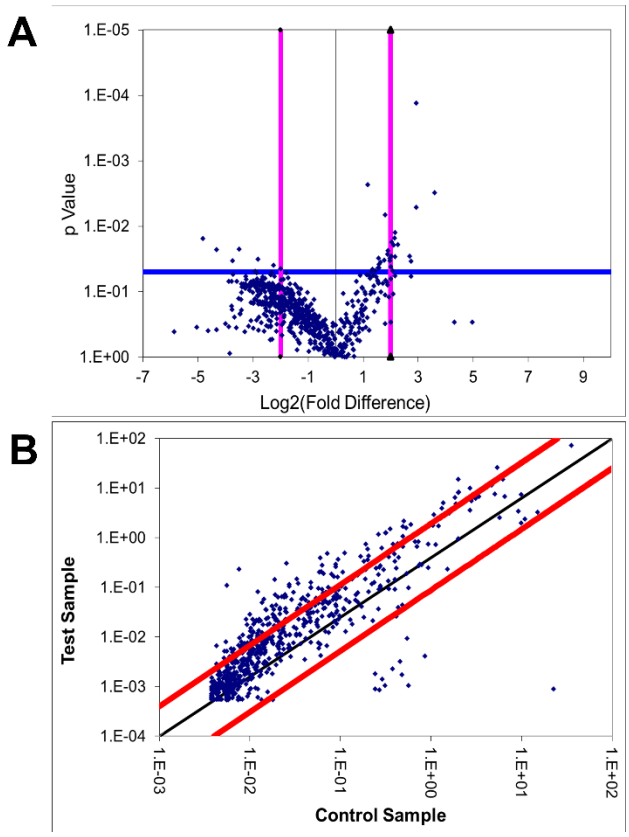
MiRNome Analysis in Endometrial Tissues

A human miRNome qPCR array consisting of primers for over 750 identified human miRNAs were used to detect changes in global miRNA expression in eutopic endometrial tissue from control women (control, n=5) and ectopic endometriotic tissues from endo women with pain (endo, n=4). Statistical analysis was performed using the online software portal available at the manufacturer's website (SA Biosciences, Valencia CA). Student's t-test (the default statistical test used by the manufacturer) showed that thirty-seven miRNAs were significantly differentially expressed ($p<0.05$) between control and endo tissues (Figures 3.1A and 3.1B). As shown in Figure 3.1C, twenty-nine of these miRNAs were upregulated in endometriotic tissues compared to controls (shown in red) while eight were downregulated (shown in green). The potential mRNA targets of the 37 significantly altered miRNAs was determined using the TargetScan Human 7.0 online database (www.targetscan.org) and Ingenuity Pathway Analysis (IPA, www.ingenuity.com), with emphasis on target genes that played a functional role in: (i) *Endometriosis* – Do these miRNAs target any genes that are already associated with the disease

state? (ii) *Pain and inflammation* –Do these miRNAs target any neuropathic or inflammatory mediators or regulators? (iii) *Epigenetic mechanisms* – Do these miRNAs target any genes associated with epigenetic markers?

TargetScan and IPA analysis narrowed the list to the following miRNAs that were closely associated with the afore-mentioned pathways: hsa-miR-29a, hsa-miR-148a, hsa-miR-100, hsa-miR-548l, and hsa-let-7g (Table 3.1). Human miR-29a, miR-148a, miR-100, and let-7g were upregulated in endometriotic tissues compared to control tissues, while the expression of miR-548l was significantly lower in endometriotic tissues than in control tissues. Each of these miRNAs has been shown to target key genes that play a role in endometriosis, pain, and/or epigenetics.

The mRNA expression of few of the miRNA target genes—B-cell lymphoma 2 (*BCL2*), DNA methyltransferase 3B (*DNMT3B*), and the mu-opioid receptor (*OPRM1)*—was measured using RT-qPCR. Compared to control tissues, the expression of *BCL2* (fold change = 0.75), *DNMT3B* (fold change = 0.57), and *OPRM1* (fold change = 0.51) were all lower in tissues from endometriosis patients.



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miRNA	Up/Down Regulation	p-value
hsa-miR-17	3.400	0.033
hsa-miR-130a	7.579	0.000
hsa-miR-30c	4.308	0.019
hsa-let-7i	3.918	0.034
hsa-miR-126	2.518	0.045
hsa-miR-30b	3.908	0.031
hsa-miR-10a	2.697	0.048
hsa-miR-29a	6.460	0.029
hsa-miR-26b	3.683	0.027
hsa-let-7g	3.450	0.007
hsa-miR-26a	4.760	0.019
hsa-miR-28-5p	2.966	0.038
hsa-miR-10b	3.727	0.024
hsa-miR-24	2.958	0.043
hsa-miR-9	4.196	0.048
hsa-miR-660	4.438	0.012
hsa-miR-148a	4.409	0.015
hsa-miR-146b-5p	6.685	0.034
hsa-miR-152	2.931	0.031
hsa-miR-103	12.075	0.003
hsa-miR-199a-3p	7.605	0.005
hsa-miR-100	4.097	0.017
hsa-miR-99a	4.056	0.029
hsa-miR-20a	4.463	0.029
hsa-miR-374b	3.986	0.041
hsa-miR-151-3p	2.473	0.048
hsa-miR-181a	2.234	0.002
hsa-miR-30e	2.896	0.043
hsa-miR-151-5p	3.094	0.036
hsa-miR-548d-5p	0.248	0.045
hsa-miR-141*	0.132	0.049
hsa-miR-518c*	0.050	0.023
hsa-miR-31*	0.074	0.034
hsa-miR-548l	0.087	0.022
hsa-miR-1227	0.209	0.040
hsa-miR-1537	0.035	0.016
hsa-miR-1538	0.142	0.032

Figure 3.1. Differentially expressed miRNAs in endometrial tissues. Significant Differentially expressed miRNAs in ectopic endometriotic tissues (endo, n=4) compared to eutopic control endometrium (control, n=5) based on the Qiagen MiRNome qPCR array. Fold change determined by SA Biosciences software. **A)** Volcano plot comparing the fold change (difference) in miRNA expression between control and endo tissues, as well as the corresponding *p*-values. Dots above the blue horizontal line indicate p>0.05. Pink vertical lines indicate 2-fold decrease and increase in expression. **B)** Scatter plot comparing control and endo tissues. The black line indicates fold changes ($2^{-\Delta Ct}$) of 1. The red lines indicate the fold-change in gene expression threshold, defined as 4. **C)** List of the 37 differentially expressed miRNAs. Red cells indicate upregulation of miRNA expression in endo tissues while green cells indicate downregulation of expression in endo tissues compared to control tissues. Significance determined by a *p*-value<0.05.

miRNA	Fold Change (Tissues, array)	GENE (pain/inflamm)	GENE (epigenetic)	GENE (endo)
hsa-miR-29a	6.46	***CNR1, CX3CL1***	*KDM5A/5C/6B/4B, PHF21A, DNMT3A,* ***DNMT3B*** ↓↓	***BCL2*** ↓
hsa-miR-148a	4.41	***PTGES3***	*SIRT1, COX1, KDM6B*	*DNMT1,* ***DNMT3B*** ↓↓
hsa-miR-100	4.10	*mTOR*		*mTOR, IG1R*
hsa-miR-548l	0.09	***OPRM1*** ↓↓		
hsa-let-7g	3.45	***NGF, OPRM1*** ↓↓, ***SCN11A, IL6***		

Table 3.1. List of miRNAs with functional role in endometriosis and/or nociception. TargetScan and IPA software analysis were used to identify target genes with functional role in inflammatory/nociceptive, epigenetic and endometriosis. Bolded genes were further investigated in this study. Fold change values were based on the Qiagen MiRNome qPCR array and associated SA Biosciences software. RT-qPCR analysis showed that compared to control tissues, the mRNA expression of *BCL2* (fold change = 0.75), *DNMT3B* (fold change = 0.57), and *OPRM1* (fold change = 0.51) were lower in endometriotic tissues, as indicated by ↓. For all tissue micronome array data, $p<0.05$.

MiRNome Analysis in Endometrial Cells Treated with Peritoneal Fluid

Since we and others have shown a prominent role for PF in pain associated with endometriosis (Ahn et al., 2015; Bedaiwy & Falcone, 2003; Kyama et al., 2009; B. D. McKinnon et al., 2015; K. Ray et al., 2015), we next determined the changes in the miRNA profile in endometrial cells treated with PF from patients with and without endometriosis and/or pain. MiRNome array showed 89 miRNAs to be differentially expressed between cells treated with PF from patients with no endometriosis (control, NN-PF) compared to PF from patients with endometriosis, with (YY-PF) and without (YN-PF) pain (Supplementary Table 3.2). It is interesting to note that there was upregulation of only two of the 89 differentially expressed miRNAs in cells treated with YY-PF. The majority (98% of YY-PF, 62% of YN-PF) of the miRNAs were downregulated when the patient had endometriosis.

MiRNome Analysis in Endometrial Cells Treated with Oxidatively-Modified Lipoproteins

We had recently shown that oxidatively modified LDLs (ox-LDLs) are powerful nociceptive mediators and are present in abundance in the PF of women with endometriosis (K.

Ray et al., 2015). We thus determined the ability of native LDL (L1) and various forms of ox-LDL preparations (minimally modified LDL-L2, oxidized LDL-L3 and fully oxidized LDL-L4) to alter the miRNA profile in endometrial cells and compared it to that seen in PF treated cells. Figure 3.2 is a Venn diagram that represents the distribution of significantly differentially expressed miRNAs in ox-LDL- and PF-treated cells.

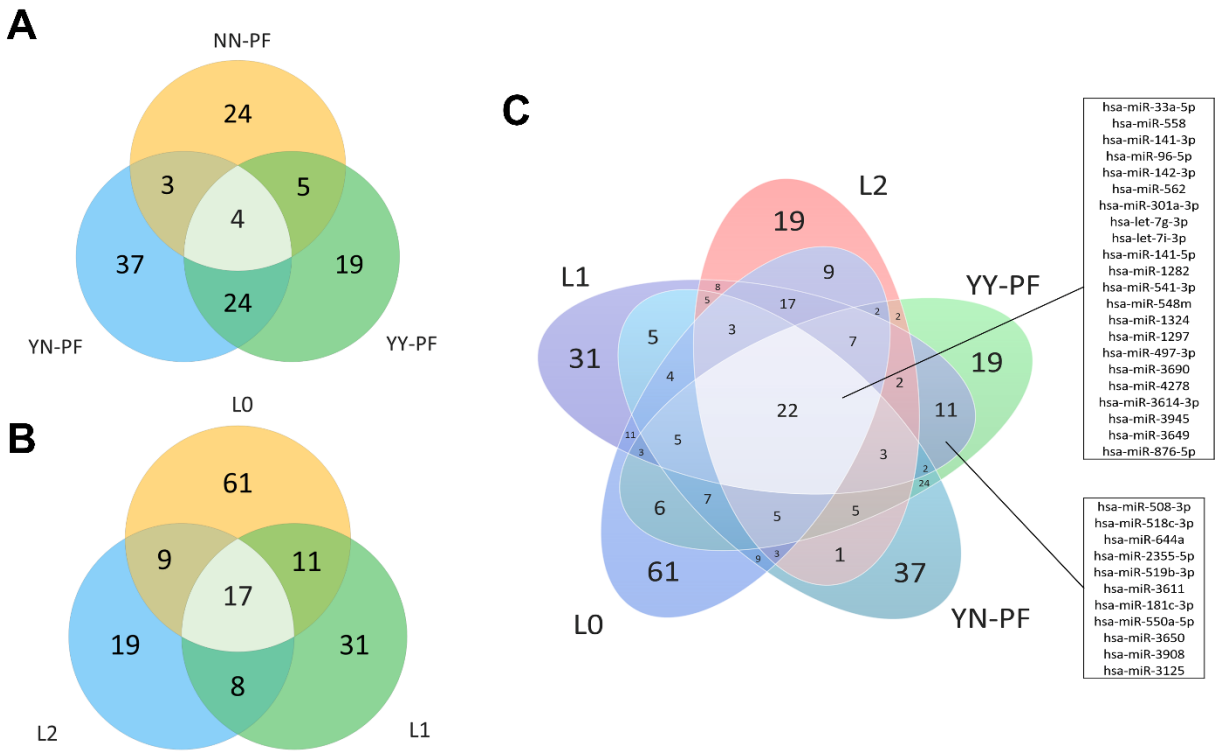


Figure 3.2. Comparison of differentially expressed miRNAs in PF and Ox-LDL treated endometrial cells. Venn diagrams indicate the numbers of miRNAs that are significantly differentially expressed in treated cells compared to media control (p<0.05). **A)** Distribution of miRNAs that were significantly differentially expressed PF-treated cells. The largest commonality (n=24) was between endo PF groups (YY-PF and YN-PF). **B)** Distribution of miRNAs that were significantly differentially expressed in ox-LDL-treated cells. **C)** Distribution of significant miRNAs in endo PF-treated and ox-LDL treated cells. Twenty-two miRNAs (listed) were significantly expressed in all treatment groups except NN-PF. Eleven miRNAs (listed) were only significant in YY-PF and L1 treated cells.

MiRNA Target Genes in PF or ox-LDL Treated Cells

To assess the potential functional relevance of the differentially expressed miRNAs in ox-LDL or PF treated cells, RT-qPCR was performed to determine the levels of target genes of

select miRNAs involved in nociceptive/inflammation pathways. Figure 3.3 shows the expression of nociceptive genes, nerve growth factor (*NGF*), cannabinoid receptor 1 (*CNR1*), and sodium voltage-gated channel alpha subunit 11 (*SCN11A*), as well as inflammatory genes interleukin 6 (*IL6*) and prostaglandin E synthase 3 (*PTGES3*) in cells treated with ox-LDLs and PF. In general, the presence of the ox-LDLs resulted in an increase in gene expression, with the ox-LDL (L2) treatment group having significantly higher expression of *NGF* ($p<0.001$), *PTGES* ($p=0.0113$), and *IL6* ($p<0.001$). No significant difference in the expression of these target genes were seen in cells treated with NN-PF and cells treated with YY-PF or YN-PF, but there was a trend towards higher expression of *CNR1* and *SCN11A* in cells treated with PF from endometriosis patients (YY-PF and YN-PF). Similar trends in gene expression were observed in the mu opioid receptor (*OPRM1*) and fractalkine ligand (*CX3CL1*). No statistical significance in expression was observed among the treatment groups, but there was a 2 to 3-fold induction of CX3CL1 by ox-LDLs.

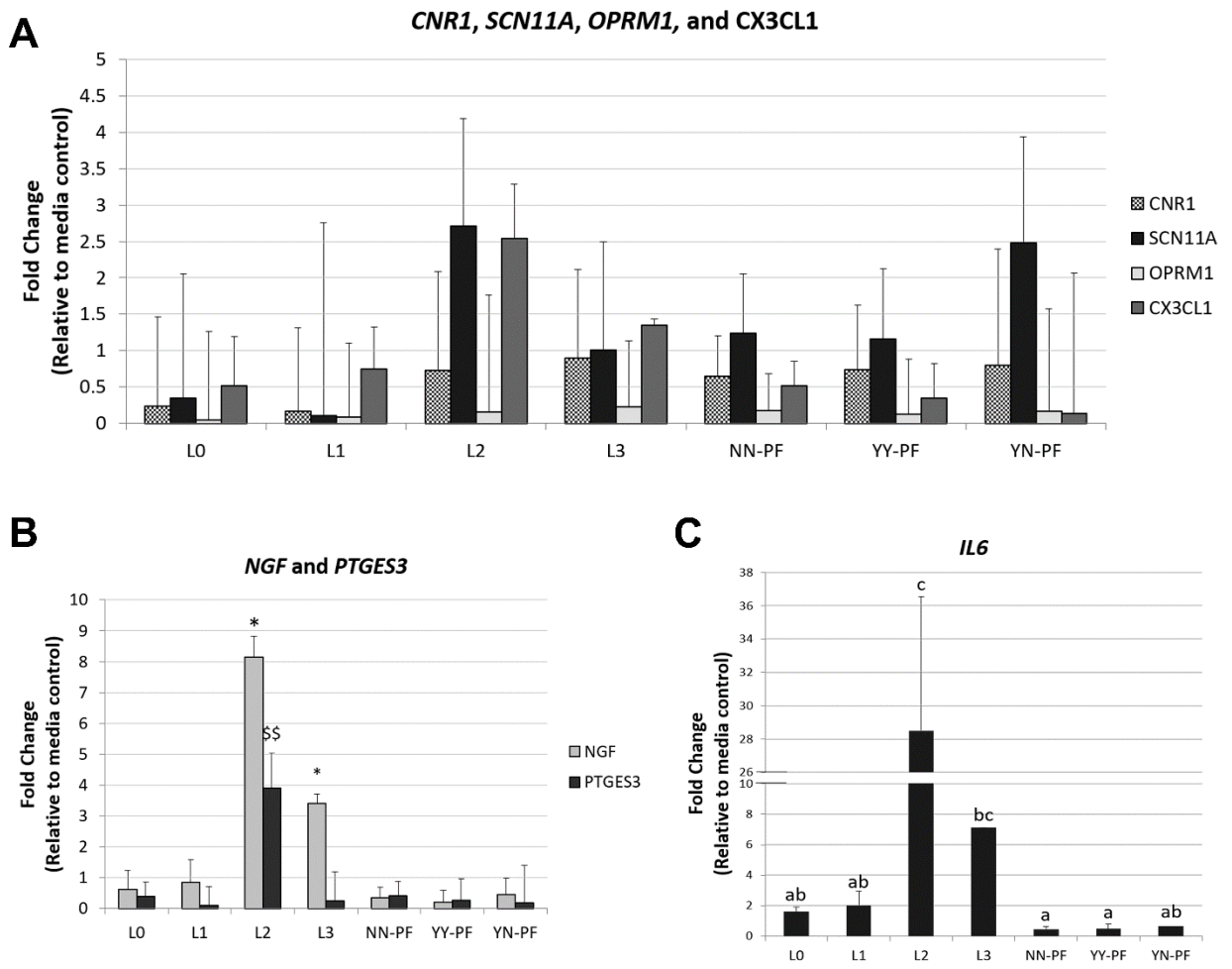


Figure 3.3. mRNA expression of miRNA targeted genes. mRNA expression of neuropathic and inflammatory target genes in endometrial cells treated with PF and oxidatively-modified LDLs as determined by RT-qPCR. **A)** Expression of *CNR1* (targeted by miR-29a), *SCN11A* (targeted by let-7g), *OPRM1* (targeted by let-7 and miR-548l), and *CX3CL1* (targeted by miR-29a). No significant differences in expression were observed with these genes. **B)** *NGF* (targeted by let-7g) was significantly differentially expressed among treatment groups (one-way ANOVA $p<0.001$). Expression in cells treated with L1 and L2 was significantly higher than expression in other treatment groups. Expression of *PTGES3* (targeted by miR-148a) across sample groups was also significant ($p=0.0113$). Treatment with L2 resulted in overexpression of *PTGES3*. **C)** Expression of *IL6* (targeted by let-7g) across sample groups ($p<0.0001$). Treatment with L3 resulted in IL6 expression that was significantly higher than the NN-PF and YY-PF treatment groups, while treatment with L2 resulted in significantly higher expression than all treatment groups other than L3.

Translational Regulation of Differentially Expressed miRNAs

MicroRNAs modulate both the transcriptional and translational levels of their target genes, thus regulating gene pathways. The protein levels of genes involved in nociceptive and inflammatory pathways were measured in endometrial cells treated with PF or ox-LDLs using the Human Neuro Discovery Array (Ray Biotech, Inc.). As shown in the heat map (Figure 3.4A), many cytokines had similar expression across all treatment groups except for MCP1 (CCL2) and monocyte inflammatory protein-Mip1α (CCL3). Densitometric analysis showed a 14.6-fold and 8.9-fold, respectively, increase in expression of MCP1 and Mip1α in cells treated with PF from patients with endometriosis and pain (YY-PF) compared to media control. Expression of MCP1 and Mip1α in YN-PF treated cells was very similar to that seen in control media (0.83-fold and 0.97-fold, respectively). These two proteins were also overexpressed by 2.78-fold and 1.08-fold in L2-treated cells, with expression trending downward as LDL oxidation increased (Figure 3.4B).

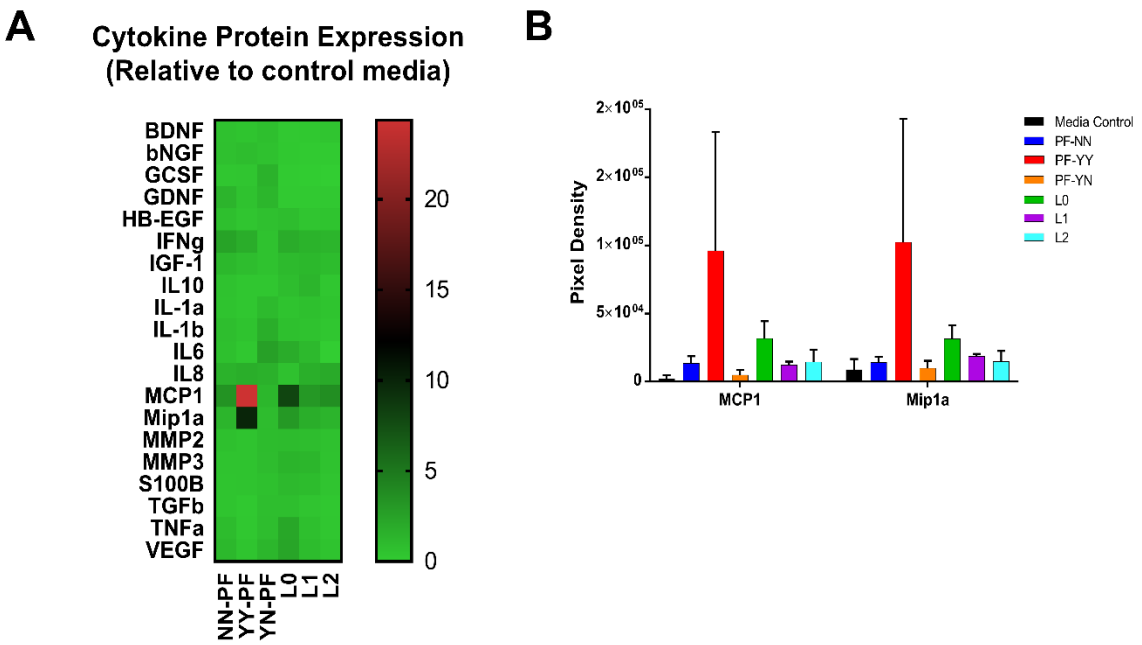
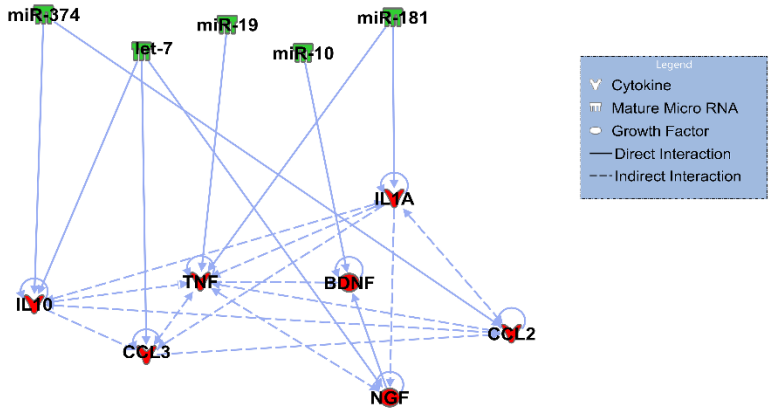


Figure 3.4. **Protein array of inflammatory and nociceptive targets.** The RayBiotech Human Neuro Discovery Array C1 was used to determine the expression of 20 human immunomodulators in treated endometrial cells. **A)** Heat map showing relative expression of inflammatory and nociceptive proteins in endometrial cells treated with PF and ox-LDLs (n=3). PF was obtained from patients with neither endometriosis nor pain (PF-NN, n = 6), with endometriosis and pain (PF-YY, n = 6), and with endometriosis and no pain (PF-YN, n = 4). **B)** Fold change ratio for MCP1 and Mip1a was calculated in comparison to media control (n = 4). While cells treated with PF-YY typically had cytokine expression that was lower than or similar to other cells treatment groups, MCP1 and Mip1α showed an exaggerated increased trend. A similar trend was seen in protein from cells treated with ox-LDLs, particularly native LDL (L0). Two-way ANOVA determined $p>0.05$.

Pathway Analysis to Identify Associations between Differentially Expressed miRNAs and Nociceptive/Inflammatory Targets

Figure 3. 5 summarizes the potential interactions between the miRNAs differentially expressed in PF and ox-LDL treated cells and their predicted targets, as determined by RT-qPCR arrays or protein array. Predicted targets of let-7 family, miRNA10 a/b, -181, -98, -19 and -374 showed association in the treated cells. Mip1α, is a documented target of let-7 (TargetScan.org), of which two isoforms (let-7i/g) were significantly downregulated in PF and ox-LDL-treated

endometrial cells. MCP1 is a target of miR-374 (IPA), whose decreased expression was only significant in the L2 cell treatment group.



Cytokine/Gene	miRNA	miRNA Expression	References for Cytokine in Endometriosis
Brain derived neurotrophic factor (BDNF)	miR-10ab		(117-119)
Interleukin 10 (IL-10)	let-7ig	↓↓	(120, 121)
Interleukin 1α (IL-1α)	miR-181c	↓	(122, 123)
Tumor necrosis factor (TNF)	miR-19		(124-126)
Nerve growth factor (NGF)	let-7ig	↓↓	(86, 127)
Macrophage inflammatory protein 1α (Mip1α/CCL3)	let-7ig, miR-98	↓↓	(16, 128)
Monocyte chemotactic protein 1 (MCP-1/CCL2)	miR-374a	↑↑	(16, 126)

Figure 3.5. **MiRNAs targeting key inflammatory molecules in endometrial cells treated with peritoneal fluid and ox-LDLs.** TargetScan and IPA analysis was used to identify associations between the inflammatory/nociceptive proteins determined using protein array and differentially expressed miRNAs as determined by the Human MiRNome array. ↓/↑ indicates significant miRNA expression in YY-PF and L1-treated cells. ↓↓/↑↑ indicates an expression change of at least 4-fold. For all noted miRNA expression differences, $p<0.05$.

DISCUSSION

The role of epigenetic mechanisms, including miRNA regulation, in endometriosis is still not completely understood and is an area of intense investigation. In the past few years, there has been a tremendous interest among endometriosis researchers to identify miRNA signatures that play a role in the pathophysiology of endometriosis. This led to a series of studies demonstrating differences in miRNA expression between paired ectopic and eutopic endometrial

tissues versus normal endometrium (Burney et al., 2009; Filigheddu et al., 2010; Hawkins et al., 2011; Laudanski et al., 2013; Ohlsson Teague et al., 2009; Pan & Chegini, 2008). The majority of these miRNAs are located in the genomically unstable sites, lending to their targeting of oncogenes, tumor suppressor genes, angiogenesis, and genes associated with inflammation or immune function (Bartel, 2004; Pan & Chegini, 2008). Functional pathway analyses of miRNA targets showed alterations in genes such as aromatase (*CYP-19*) and *COX-2* as well as those involved in apoptosis and cell-signaling to be differentially expressed in endometriosis (Hirakawa et al., 2016; Long, Wan, La, Gong, & Cai, 2015; Mari-Alexandre et al., 2015; Ohlsson Teague et al., 2009; Okamoto et al., 2015). For example, the downregulation of migration inhibitory factor (MIF) in ectopic endometriotic lesions compared to eutopic endometrium has been attributed to the upregulation of miR-451(Graham, Falcone, & Nothnick, 2015). Similarly, miR-93, which targets MMP3 and VEGFA, genes involved in angiogenesis and shown to inhibit proliferation, invasiveness, and migration of endometrial stromal cells, was underexpressed in ectopic tissues when compared to control endometrium (Lv, Chen, & Liu, 2015). Validation studies using these tissues or isolated primary endometrial cells showed that several of these miRNAs were influenced by ovarian steroids (Nothnick & Healy, 2010; Pan, Luo, Toloubeydokhti, & Chegini, 2007; Toloubeydokhti, Pan, Luo, Bukulmez, & Chegini, 2008). Though these studies speculated the association between the differentially expressed miRNAs to pathophysiological changes in endometriosis, none of these studies directly delved into whether any of these miRNAs may be playing a regulatory role in pain associated with endometriosis.

There are very few studies in the literature that have shown a direct relationship between miRNA changes and pain (Bai, Ambalavanar, Wei, & Dessem, 2007; Bezerra, Lima, Girao,

Teixeira, & Graca, 2008; Burney et al., 2009; Chehade & Sampson, 2008; Sanchez Freire et al., 2010). Bai et al recently showed down-regulation of several miRNAs in the trigeminal ganglion neurons following inflammatory muscle pain (Bai et al., 2007). Recent investigations have shown that expression of miR-100 and miR-29a in tissues of the central nervous system (spinal cord and dorsal root ganglion, respectively) are associated with neuropathic and inflammatory pain in animal models (Kynast, Russe, Geisslinger, & Niederberger, 2013; Qureshi et al., 2015). In our study, we observed that both these miRNAs were significantly upregulated in ectopic lesions compared to the control tissues, along with 27 other miRNAs. Additionally, we found that mRNA targets of these miRNAs-*BCL2, DNMT3B,* and *OPRM1*-were also downregulated in the endometriotic tissues.

The peritoneal milieu in women with endometriosis expresses several mediators, such as PGE2, that play an important role in pain (Barcena de Arellano et al., 2011; Barcena de Arellano & Mechsner, 2014; Morotti et al., 2014; Neziri et al., 2014; Santanam et al., 2013). The cyclooxygenase (COX-2) enzymes that synthesize prostaglandins are highly expressed in endometriotic glands (Hayashi et al., 2013; Jana, Chatterjee, Ray, DasMahapatra, & Swarnakar, 2016; Kilico, Kokcu, Kefeli, & Kandemir, 2014; Y. Liu et al., 2011) and their increased expression strongly correlates with pathological abnormalities (Buchweitz et al., 2006; Cho et al., 2010; H. Y. Kim et al., 2012; H. Wang, Sun, Jiang, Liu, & Wang, 2016). However, the contribution of the PF milieu to the observed miRNA changes during endometriosis is not clearly known. In a recent study, exposure of primary (eutopic and ectopic) cells to PF from endometriosis patients compared to control patients, caused lower expression of a number of miRNAs that played a role in angiogenesis (e.g. VEGFA) (Braza-Boils et al., 2015). Our study showed similar trends in relation to miRNAs that target inflammation and nociceptive pathways.

We did not observe significant overlap between miRNAs altered in PF-treated cells and those that were differentially expressed in eutopic/ectopic endometriotic tissues. This lack of overlap might be related to time of exposure to PF. Most of our cell treatments are 48 hour exposures; however, the endometriotic tissues obtained from patients have been exposed to PF for months or even years. However, endo PF or ox-LDL treated cells seemed to cause a global downregulation of miRNA expression compared to untreated cells. Additionally, there were many miRNAs that were similarly (up or down) regulated in cells treated with endo PF and those treated with ox-LDLs (Figure 3.2). The ox-LDL treatment also produced significant overexpression of nociceptive and inflammatory genes *NGF, PTGES3,* and *IL6*. While it did not reach significance, the expression of fractalkine (*CX3CL1*) and *OPRM1* was of interest due to the established association between these genes and the progression of endometriosis (Hou et al., 2016; Shimoya et al., 2005; Y. Wang et al., 2014).

Pathway analysis using TargetScan and IPA analysis identified protein targets of the differentially expressed inflammatory and nociceptive genes, such as the induction of MCP-1 by *IL-6* (Arendt et al., 2002; Biswas et al., 1998; Choi, Rotimi, O'Carroll, & Nicholson, 2016). MCP-1, along with Mip-1α, was highly expressed in cells treated with ox-LDLs and YY-PF. Both of these signaling proteins attract macrophages and monocytes to the site of inflammation (Na et al., 2011; Wickstrom, Stavreus-Evers, Vercauteren, Olovsson, & Edelstam, 2017). Both these inflammatory molecules are also associated with nociception (Dauvergne et al., 2014; Kwiatkowski et al., 2016; Menetski et al., 2007). Immunoblotting indicated that ox-LDLs may increase the level of MCP-1 protein in treated cells similar to endo PF treatment, supporting our paradigm that the LDL components of the PF is responsible for the modulation of these chemokines and play a key role in nociception. Additionally, miR-374, which targets MCP-1,

was also significantly downregulated in cells treated with oxidatively-modified LDLs (L2), further validating our findings.

Pathway analysis also identified potential relationships among the miRNAs that were modulated by PF or ox-LDL treatments and their predicted target genes (Figure 3.5). A higher frequency of down-regulation of the let-7 family of miRNAs was seen. This is not surprising when we consider several evidences in cancer research that let-7 is a potential tumor suppressor whose altered regulation leads to many types of cancer (C. H. Tsai et al., 2015; Yang, Zhang, Dong, Chang, & He, 2012). The let-7 family also targets opioid receptors and other nociceptors (He & Wang, 2012; He, Yang, Kirkmire, & Wang, 2010; Park et al., 2014). It was recently shown that the let-7 cluster of miRNAs plays a role in endometriosis. Circulating let-7 isoforms have been reported at varying levels in endometriosis patients (Seifer, Su, & Taylor, 2016). Studies have shown that the let-7 cluster is regulated by ox-LDLs (Ding, Wang, Khaidakov, Liu, & Mehta, 2012; B. Qin et al., 2012; Tang et al., 2015). Increased expression of the let-7f isoform in endometrial cells decreased cell migration (Cho et al., 2016). While the previous study only investigated isoforms a-f, our finding that PF and ox-LDL treatments can also modulate let-7i-g suggest oxidative components of patient PF may also potentially play a role in endometrial cell invasion and migration.

CONCLUSIONS

Endometriosis is a debilitating, chronic inflammatory condition that afflicts many young women around the world with chronic pain. Knowledge of pathways involved in the pathophysiology of endometriotic pain and regulators of such pathways will be a great asset in identifying new and appropriate targets for therapy. MiRNAs have established themselves as critical epigenetic regulators in the development and progression of several diseases, including

endometriosis. MiRNAs can regulate several nodal points in the complex etiology of endometriosis and its associated pain. Our data confirms earlier findings (Burney et al., 2009; Ohlsson Teague et al., 2009; Pan & Chegini, 2008; Pan et al., 2007; Teague et al., 2010) that miRNAs are down-regulated in endometriosis, but also additionally provide evidence that the presence or absence of pain discriminates the miRNA signature in these women. Our studies therefore suggest pain symptoms to be a unique discriminator of miRNA fingerprint in endometriotic women.

Our observation that Ox-LDL treated cells have very similar response on miRNA profile to the endo PF treatment suggests that many of these pain-targeting miRNAs are oxidation sensitive and can be targeted by drugs that reduce oxidation. MiRNA-based therapeutics provide a possible novel way to treat endometriotic symptoms. A potential example is the let-7 cluster, which is a known tumor suppressor and apparent target of oxidative stress in the peritoneal cavity of endometriosis patients. However, the ubiquitous nature of the let-7 family and their role in cellular homeostasis makes this option extremely complex. Over the past several years, researchers in cancer biology have made key advancements toward a let-7 therapy for various cancers, but the balancing act requires extensive preliminary studies in cell and animal models (Ciarmela et al., 2013; Young et al., 2013). Based on our findings, another option would be to target the other miRNAs shown in Figure 3.5, for which validations studies need to be conducted. Lastly, our findings also support the potential use of agents that will diminish the oxidative stress in the peritoneal cavity (ox-LDLs), thus alleviating chronic pelvic pain.

ACKNOWLEDGEMENTS

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GENE NAME	PRIMER TYPE	SEQUENCE
CNR1	sense	5′- GGCACCCAGAAGAGCATCAT -3′
	antisense	5′- GGACCAGGGTCTTGGCTAAC -3′
NGF	sense	5′- GGAGCGCAGCGAGTTTTGG -3
	antisense	5′- GCTGCTCCCTTGGTAAAACTG -3′
SCN11A	sense	5′- CAGGCTGTTTTATTCCCGCC -3′
	antisense	5′- GGAAATTCCGCTCATCTGGA -3′
PTGES3	sense	5′- TCCAAGCATAAAAGAACGGACA -3′
	antisense	5′- TGTCTTGTGAATCCTTTGCCCT -3′
IL6	sense	5′- GCCAGAGCTGTGCAGATGAG -3′
	antisense	5′- TGGCATTTGTGGTTGGGTCA -3′
OPRM1	sense	5′- AACACTCATGGTGCAGAGGG -3′
	antisense	5′- TGGGAACATGGCCATTTGGA -3′
CX3CL1	sense	5′- TCATGTGTGAGATGGGAGGA -3′
	antisense	5′- TTGACCATTCTCCACCTTCC-3’
DNMTB3	sense	5’- CTGCCACAAGACAAACAGCC -3’
	antisense	5’- GCAAAGACCGAGGGGATGAA -3’
BCL2	sense	5’- GAAATCAAACAGAGGCCGCAT -3’
	antisense	5’- ACTGAGTACCTGAACCGGCA -3’

Supplementary Table 3.1. **Primer sequences for RT-qPCR analysis of nociceptive gene expression in PF and ox-LDL-treated cells.** Primers were designed using NCBI GenBank and ordered from Invitrogen.

	YY-PF		YN-PF	
miRNA	Fold Change	p-value	Fold Change	p-value
hsa-miR-643	1.7204	**0.04741**	1.8397	**0.036576**
hsa-miR-501-5p	0.7843	**0.02069**	0.7052	0.165759
hsa-miR-505-3p	0.7628	**0.006637**	0.6444	**0.001685**
hsa-miR-10b-5p	0.8348	**0.024119**	0.6939	0.196253
hsa-miR-576-5p	0.742	**0.004089**	0.4484	**0.040253**
hsa-miR-455-5p	0.8157	**0.017716**	0.5369	**0.000612**
hsa-miR-548b-3p	0.8008	**0.026477**	0.5702	**0.019257**
hsa-miR-650	0.5795	**0.016601**	2.3556	0.388946
hsa-miR-551a	0.8045	**0.041577**	0.7369	0.201531
hsa-let-7f-5p	0.7897	**0.028139**	0.7003	0.280162
hsa-miR-34a-5p	0.7593	**0.013057**	0.5795	**0.001786**
hsa-miR-182-5p	0.8622	**0.028128**	0.7217	0.242451
hsa-miR-662	0.4567	**0.0375**	0.5662	0.216891
hsa-miR-138-5p	0.7699	**0.026367**	0.6385	**0.017571**
hsa-miR-27b-3p	0.7506	**0.013536**	0.561	**0.012939**
hsa-miR-519e-5p	0.5768	**0.022166**	0.4536	**0.008043**
hsa-miR-587	0.6749	**0.037435**	1.277	0.23306
hsa-miR-590-5p	0.6595	**0.032845**	0.4873	0.006277
hsa-miR-515-5p	0.7167	**0.009601**	0.7437	0.053605
hsa-miR-885-5p	0.6055	**0.044622**	0.7284	**0.047839**
hsa-miR-20a-5p	0.7681	**0.006582**	0.5495	**0.042996**
hsa-miR-98-5p	0.8101	**0.026647**	0.6656	0.075716
hsa-miR-520g-3p	0.7489	**0.042787**	0.6055	**0.021842**
hsa-miR-301a-3p	0.742	**0.032235**	0.4941	**0.005763**
hsa-miR-137	0.5419	**0.029349**	0.6971	0.066959
hsa-miR-875-3p	0.6549	**0.038153**	0.6796	**0.038098**
hsa-miR-339-5p	0.8195	**0.00762**	0.8119	0.255393
hsa-let-7f-1-3p	0.6097	**0.007966**	0.3084	**0.004875**
hsa-let-7i-3p	0.6796	**0.047718**	0.5623	**0.017681**
hsa-miR-200a-3p	0.7343	**0.04361**	0.75	0.085695
hsa-miR-1305	0.6664	**0.028211**	0.5267	0.183283
hsa-miR-29c-5p	0.8017	**0.048458**	0.687	0.110165
hsa-miR-328-3p	0.6006	**0.047724**	0.612	0.287449
hsa-miR-139-3p	0.6726	**0.035939**	0.4328	0.154402
hsa-miR-425-3p	0.7744	**0.003428**	0.738	0.175157
hsa-miR-490-3p	0.6482	**0.03848**	0.6697	0.104849
hsa-miR-509-5p	0.4833	**0.014774**	0.325	**0.006927**
hsa-miR-219a-1-3p	0.6452	**0.048819**	0.6713	0.38213
hsa-miR-875-5p	0.5896	**0.021613**	0.6744	**0.028896**
hsa-miR-1285-3p	0.6618	**0.0201**	0.8226	0.288655
hsa-miR-1286	0.451	**0.033552**	0.4903	**0.00863**
hsa-miR-1289	0.2725	**0.041345**	0.4142	0.102944
hsa-miR-1178-3p	0.5425	**0.028634**	0.6206	**0.039892**
hsa-miR-1182	0.5501	**0.024954**	0.6934	0.095629

	YY-PF		YN-PF	
miRNA	Fold Change	p-value	Fold Change	p-value
hsa-miR-1207-5p	0.5539	**0.026429**	0.7112	0.293818
hsa-miR-185-3p	0.6452	**0.011995**	0.9559	0.833009
hsa-miR-541-5p	0.4615	**0.047741**	0.6177	0.308559
hsa-miR-548i	0.5204	**0.024808**	0.6636	0.071531
hsa-miR-548o-3p	0.7275	**0.044601**	0.5377	0.162294
hsa-miR-654-3p	0.6006	**0.025847**	0.687	**0.035915**
hsa-miR-675-5p	0.629	**0.043935**	0.4532	**0.012699**
hsa-miR-675-3p	0.706	**0.03347**	0.659	0.050025
hsa-miR-1295a	0.6348	**0.013085**	0.7014	**0.042299**
hsa-miR-1263	0.5216	**0.043909**	0.5967	0.061184
hsa-miR-516a-5p	0.6726	**0.015448**	0.6307	**0.019225**
hsa-miR-3131	0.5748	**0.04174**	0.7448	0.486365
hsa-miR-30d-3p	0.7077	**0.010209**	0.5804	**0.01183**
hsa-miR-644a	0.622	**0.026143**	17.5695	0.373936
hsa-miR-3173-3p	0.2603	**0.036042**	12.3092	0.373951
hsa-miR-302e	0.5844	**0.01414**	18.0217	0.373933
hsa-miR-642b-3p	0.4429	**0.003427**	16.971	0.373741
hsa-miR-617	0.3326	**0.020539**	11.3006	0.374
hsa-miR-4295	0.5645	**0.017558**	15.9446	0.373949
hsa-miR-3667-3p	0.5817	**0.015031**	16.0185	0.373948
hsa-miR-3659	0.5218	**0.046734**	18.1052	0.373925
hsa-miR-3199	0.571	**0.01576**	16.1299	0.373947
hsa-miR-3616-5p	0.5279	**0.001634**	14.6382	0.373962
hsa-miR-4273	0.4564	**0.025758**	13.0412	0.37398
hsa-miR-654-5p	0.5554	**0.017563**	17.8973	0.373923
hsa-miR-4283	0.7891	**0.0458**	31.523	0.373902
hsa-miR-3654	0.4736	**0.024624**	8.8254	0.373947
hsa-miR-887-3p	0.6514	**0.047657**	20.3224	0.373924
hsa-miR-3917	1.1238	**0.035292**	24.6754	0.373908
hsa-miR-4302	0.5763	**0.020225**	21.9833	0.373893
hsa-miR-3132	0.4983	**0.048157**	13.4388	0.373963
hsa-miR-449c-3p	0.4835	**0.024613**	15.1544	0.373954
hsa-miR-214-5p	0.605	**0.025675**	22.6536	0.373904
hsa-miR-3691-5p	0.3372	**0.012973**	6.416	0.374053
hsa-miR-3201	0.5606	**0.033953**	17.0103	0.373939
hsa-miR-586	0.5567	**0.020582**	15.7251	0.373951
hsa-miR-4265	0.5353	**0.0353**	26.9398	0.373904
hsa-miR-3184-5p	0.6191	**0.002646**	11.6992	0.374007
hsa-miR-3125	0.6514	**0.044602**	16.6217	0.373944
hsa-miR-4251	0.3937	**0.020965**	20.8455	0.373887
hsa-miR-3675-3p	0.6575	**0.025872**	21.3328	0.373902
hsa-let-7e-3p	0.6934	**0.024243**	15.5805	0.373948
hsa-miR-3155b	0.3664	**0.035366**	6.5508	0.374223
hsa-miR-3689e	0.2615	**0.031564**	6.829	0.374268

Supplementary Table 3.2. Significant miRNAs in endometrial cells treated with peritoneal fluid (PF) from women with and without endometriosis. YY indicates that the peritoneal fluid was from patients with endometriosis and pain (n=3). YN indicates that the patients had endometriosis but lacked pain (n=3). Significance (indicated in **bold**) was determined by a *p*-value<0.05 compared to cells treated with PF from control patients (NN-PF, n=3). Fold change determined by SA Biosciences software. Red cells indicate upregulation while green cells indicate downregulation compared to control PF

CHAPTER 4

EPIGENETIC CROSS-TALK AND REGULATION OF ENDOMETRIOSIS-ASSOCIATED PAIN

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ABSTRACT

Chronic pelvic pain associated with endometriosis has been attributed to hormonal and immune aberrations that alter the makeup of peritoneal fluid (PF) and impact surrounding tissues. It has been determined that the trimethylation of histone 3 lysine 27 (H3K27me3) plays a role in endometriosis. This histone modification has only one known methyltransferase, EZH2, the catalytic subunit of the epigenetic complex PRC2. We hypothesized that regulation of the PRC2 complex ultimately results in increased endometriosis-associated pain, potentially mediated by epigenetic events. Samples from IRB-approved and consented patients with and without endometriosis or pain were used. mRNA expression of PRC2 components was measured in endometriosis and control tissues using RT-qPCR, along with *Jumonji protein 2* and *FOXP3*, which are thought to interact with the complex. miRNA qPCR assays were used to measure the expression of miRNAs (e.g. miR-155-5p) that target the jumonji protein. Protein expression of these key regulators was determined using Western blots. Ishikawa cells treated with PF from patients with and without endometriosis and pain were used to stimulate endometrial cells. mRNA and protein expression of the afore-mentioned mediators were measured, as was the level of H3K27 trimethylation. The effects of miR-155 on jumonji protein 2 and PRC2 were determined by transfecting the cell model with a miR-155 mimic and antagonist. Cross-talk between Jumonji protein 2 and these epigenetic factors was explored using ChIP-qPCR. Our

study supported the notion that EZH2 is overexpressed in endometriosis and may contribute to the chronic pain associated with the disorder. Targeting of Jumonji protein 2 by miR-155 and hypermethylation of CpG islands in the promoter of FOXP3 likely contribute to this trend. Blocking miR-155 resulted in underexpression of EZH2 in cells treated with PF from women with endometriosis and pain ($p=0.006$) but failed to stunt expression when cells were treated with PF from endometriosis patients without pain. ChIP-qPCR potentially identified an alternate route to PRC2 and EZH2 stimulation in cells treated with PF from endometriosis patients with pain. These findings support the potential use of EZH2 inhibitors to treat endometriosis-associated pain. They also present upstream mediators, Jumonji protein 2 and miR-155, that could be therapeutically targeted.

INTRODUCTION

Endometriosis and Epigenetics

Endometriosis is defined by the presence of endometrial tissue in ectopic locations, typically in or around the peritoneal cavity (Burney & Giudice, 2012; Giudice, 2010). While the exact prevalence of endometriosis is likely underrepresented, most sources cite a minimum of 10% of women in their reproductive years have this disease (Ciarmela et al., 2013; Platteeuw & D'Hooghe, 2014). Primarily described as a hormonal disorder, the pathogenesis of endometriosis has also been linked to immunological/inflammatory, genetic, and environmental factors. More recently, the role of epigenetics in the development and progression of the disorder has been investigated (Colon-Caraballo, Monteiro, & Flores, 2015; S. W. Guo, 2009; Nasu et al., 2011; Stephens, Whitehouse, & Polley, 2013). Epigenetic mechanisms are heritable changes to one's phenotype that are not associated with a change in nucleotide sequence and include DNA

methylation, post-translational modifications to histone proteins, and often microRNAs (Bird, 2007).

Endometriosis and PRC2

In addition to heterochromatin-like protein 1 (HP-1), polycomb (PcG) and trithorax (TrxG) complexes are at the heart of epigenetics. Responsible for maintaining gene repression and activity, respectively (Steffen & Ringrose, 2014), the latter two complexes function antagonistically to establish epigenetic regulation (Steffen & Ringrose, 2014). Polycomb repressive complex 1 (PRC1), polycomb repressive complex 2 (PRC2), and PhoRC all form the PcG complexes, with the former two typically being the subject of extensive epigenetic research. The Polycomb Repressive Complex 2 (PRC2) consists of four core proteins, RbAp46/48, Embryonic Ectoderm Development (EED), Suppressor of Zeste 12 (SUZ12), and Enhancer of Zeste Homolog 2 (EZH2), the catalytic subunit of the PRC2 complex. These components work together to regulate chromatin structure via tri-methylation of lysine 27 on histone 3 (H3K27me3) (R. Cao et al., 2002; Kuzmichev, Nishioka, Erdjument-Bromage, Tempst, & Reinberg, 2002), which is also known to interact with PRC1. EED binds the histone site while EZH2 methylates it, with the help of SUZ12 (Geisler & Paro, 2015). This modification leads to the formation of closed chromatin structure (heterochromatin) and thus marks transcriptional repression, as further demonstrated by the presence of other co-factors (Fuks, 2005; Kondo, 2009; Vire et al., 2006). Little is known about the mechanistic role of this complex in the pathophysiology of endometriosis. One recent study in endometriosis showed heightened expression of EZH2 and trimethylation of H3K27 in secretory endometrium and endometriotic lesions (Colon-Caraballo et al., 2015). Additionally, an endometriosis cell culture study by Arosh and colleagues proved that inhibition of PGE2 receptors EP3 and EP4 are coincident with

decreased EZH2 expression (Arosh et al., 2015), supporting a role for PRC2 in endometriosis-associated pain.

Jumonji Proteins

It has been shown that the PRC2 complex (specifically EZH2) is, at least partly, regulated by Jumonji and AT-Rich Interaction Domain Containing 2 (G. Li et al., 2010), a member of the Jumonji family of histone demethylases and common target of differentially expressed miRNAs (miR-30b, miR-30c, miR-10a, miR-29a, miR-26a, miR-148a, miR-181a, miR-30e) in endometriotic lesions compared to control tissues (Wright 2017, accepted for publication in *Redox Biology*). The jumonji family is the largest family of histone demethylases, and all but Jumonji protein 2 contain the catalytic JmjC domain responsible for histone demethylation (Klose, Kallin, & Zhang, 2006; Kooistra & Helin, 2012). Due to its cross-talk with EZH2 and PRC2 activity in embryonic stem cells, Jumonji protein 2 is thought to be crucial in development and potentially in the progression of cancer (Landeira & Fisher, 2011; G. Li et al., 2010; Pasini et al., 2010). As such, targeting Jumonji protein 2 via modulators such as microRNAs could be one method of stunting cell proliferation. In a study of acute lymphoid leukemia, Palma and colleagues determined that miR-155-5p induced cell death via a network of mechanisms, including regulation of cyclinD1 by Jumonji protein 2 (Palma et al., 2014). Others believe that miR-155-5p could have evolved to regulate PRC2 by tweaking Jumonji protein 2 expression (Escobar et al., 2014). Interestingly, miR-155-5p is an established promoter of inflammation via regulation of macrophages and cytokines (Escobar et al., 2014; Jablonski, Gaudet, Amici, Popovich, & Guerau-de-Arellano, 2016; Yao, Li, Wu, Zhang, & Wang, 2015).

Endometriosis and FoxP3

miR-155 is highly expressed in regulatory T-cells (Tregs), where it is targeted by the transcription factor FOXP3, a known tumor suppressor. Though limited in evidence, FOXP3 plays a role in the inflammatory aspect of endometriosis. The prevalence of $FOXP3^{+}$ Tregs in an endometriotic environment during secretory phase prevented leukocyte recruitment to the sites of endometriosis (Berbic & Fraser, 2011). Additionally, PF from women with endometriosis has a higher concentration of FOXP3-expressing $TCD4^{+}CD25^{high}$cells than the PF of control patients (Olkowska-Truchanowicz et al., 2013; Podgaec, Rizzo, Fernandes, Baracat, & Abrao, 2012). Over the past several years, there has been a lack of consistent findings regarding FOXP3 expression in endometriotic lesions and eutopic tissues of endometriosis patients (Basta et al., 2010; Berbic et al., 2010). Most recently, Podgaec and colleagues found high FOXP3 expression in deep rectosigmoid lesions but lower expression in the eutopic endometrium of endometriosis patients with chronic pelvic pain (Podgaec et al., 2014). These studies provide further evidence that the immunological aspects of endometriosis are at least partly responsible for endometriosis-associated pain.

It is important to note that FOXP3 also has an indirect relationship with the EZH2 component of PRC2. In breast cancer models, overexpression of FOXP3 protein not only lessened the proliferative effects of EZH2, but also enhanced degradation of EZH2 protein (Z. Shen et al., 2013). Conversely, there is evidence that trimethylation of H3K27 by EZH2 is capable of silencing FOXP3 promoter regions, therefore leading to aberrant Treg cell differentiation and function (Xiong et al., 2012). These studies suggest a complex interplay between epigenetic mediators, PRC2 complex, miR-155-5p and the inflammatory mediator FOXP3. We hypothesized that the imbalance in this cross-talk triggers inflammatory responses

and nociception in endometriosis. The current study determined the cross-talk between these mediators in patient tissues and an endometriosis cell model.

MATERIALS AND METHODS

Human Subject Participants

Women ages 21 to 60 years, undergoing tubal ligation or having non-endometriosis disorders (controls) or patients with endometriosis ("endo", laparoscopically diagnosed followed by pathological confirmation and/or patients with symptoms) were recruited from Obstetrics-Gynecology clinic at Cabell Huntington Hospital, Joan C Edwards School of Medicine, Marshall University, in Huntington, WV. In this study, endo patients were diagnosed with stage I/II peritoneal endometriosis. This HIPAA compliant study was approved by the Institutional Review Board of the Marshall University School of Medicine and was carried out per the principles of the Declaration of Helsinki. All patients were consented prior to the study. All women completed a gynecologic/infertility history form, a pre-operative quality of life questionnaire and assessment of pain using a visual analog scale for assessment of endometriosis associated pain (dysmenorrhea, non-menstrual pelvic pain, dyspareunia, and dyschesia) (adapted from the validated International Pelvic Pain Society's Pelvic Assessment Form). Date of their last menstrual period was used to assess their cycle time. The inclusion criteria included women ages 21-60 years old, with normal menstrual cycles and otherwise in normal health (except for pain and endometriosis) who have not been on any hormonal medication for at least one month before sample collection. Exclusion criteria included subjects with current medical illnesses such as diabetes, cardiovascular disease, hyperlipedemia, hypertension, systemic lupus erythematosis or rheumatologic disease, positive HIV/AIDS, active infection. Subjects were asked to stop

multivitamins that contain high levels of antioxidants and anti-inflammatory medications one month prior to sample collection.

RNA/Protein Isolation in Peritoneal Fluid-treated Cells

Peritoneal fluid (PF) (devoid of blood contamination) was collected on ice from all women during laparoscopic surgery. Peritoneal fluid was spun at 2000xg to remove any cellular debris. The supernatant was used immediately for studies or stored in a -80^{0}C freezer for future use. To establish a cell model of the peritoneal environment, Ishikawa cells a human (39-year-old woman) established endometrial cell line (Sigma-Aldrich, St. Louis, MO), were cultured in T75 flasks in complete media (DMEM/F12, 10% FBS, 1% Pen/Strep, 1% L-glutamine). These cells were used because they express characteristics similar to those of mature endometrial epithelial cells (Bulun et al., 2006; Cho et al., 2016; Nishida et al., 1985). Approximately 70% confluent cells were treated with 1% PF from patients for 48 hours in a DMEM/F12 media containing 1% charcoal-stripped FBS. Patient peritoneal fluid (PF) groups were +endo/+pain (YY-PF), +endo/-pain (YN-PF), and –endo/-pain (NN-PF, "control fluid"). The concentrations chosen were based on our previous published studies (K. Ray et al., 2015). At the end of the 48-hour treatment, cells were collected using Qiazol Lysis reagent (Qiagen, Gaithersburg, MD) and RNA was isolated using the Qiagen miRNeasy Mini Kit. The quantity and quality of RNA were measured in the NanoDrop 2000 spectrophotometer. Cells were also collected in RIPA buffer with protease inhibitors (Thermo Scientific, USA) and protein concentrations were measured using a modified Lowry protocol (Lowry et al., 1951)

Endometrial Tissue Collection and RNA/Protein Isolation

Endometrial (eutopic) tissues from control patients (EuNN), eutopic tissues from endometriosis (ovarian or peritoneal endometriosis, "endo") patients (EuYY), and ectopic

endometriotic tissues (EcYY) from endo patients were removed during laparoscopy/laparotomy by a qualified physician. Biopsy fragments were immediately placed in RNA*later* solution (Qiagen) and subsequently stored in a freezer at -80°C. RNA extraction from 100 mg of tissue (eutopic and ectopic) was carried out using Qiazol Lysis Reagent (Qiagen). Tissues were homogenized using zirconium oxide beads in a Bullet Blender® homogenizer (Next Advance, USA) and RNA was isolated using the Qiagen miRNeasy Mini Kit following the manufacturer's recommendations. The quantity and quality of RNA were measured in the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Similarly, 50 mg of tissue was homogenized in RIPA buffer prior to protein estimation by a modified Lowry method.

mRNA Expression in Tissues and PF-treated Ishikawa Cells

RNA (which includes miRNA) isolated from the tissues and treated cells were used. cDNA synthesis from 1 µg of each sample was performed using iScript RT II Kit (Qiagen). mRNA expression was analyzed in the cDNA samples using SYBR Green (Biorad, Hercules, CA) and the primers listed in Supplementary Table 4.1. cDNA synthesis from 2 µg of each sample was performed using miScript RT Kit (Qiagen). To determine the expression of miR-29a, miR-148a, and miR-155 in tissues and PF-treated cells, the appropriate Qiagen Primer Assay Kit was used, following the manufacturer's protocol for qPCR. A primer assay for RNU6 was used for a qPCR housekeeping gene.

Protein Expression in PF-treated Cells and Patient Tissues

Total protein was measured using a modified Lowry method. Protein (35µg) was separated on a 4-20% Tris-HCl gradient gel (Biorad) and transferred onto nitrocellulose membranes. After washing with Tris-buffered saline with Tween 20 (TBST), the membranes were blocked in 5% bovine serum albumin or 5% milk in TBST for 1 hour, then incubated at

4°C overnight with anti-rabbit antibody against Jumonji protein 2, FOXP3, EZH2, and H3K27me3 (1:1000, Cell Signaling, Danvers, MA) and anti-mouse against β-actin (1:4000, Sigma-Aldrich). Anti-rabbit antibody against H4 was diluted 1:20000. The membranes were washed and incubated with HRP-linked anti-rabbit or anti-mouse secondary antibody (1:6000, Sigma-Aldrich, St. Louis, MO) for one hour at room temperature. After washing, membranes were developed in HRP Substrate (Millipore, Temecula, CA) and imaged using the ChemiDoc system (Biorad). Densitometric levels of protein bands were quantified and expressed as their relative ratio to β-actin.

Cell Transfection with miR-155 Mimic/Antagonist

Cells were transfected using SiPORT™ NeoFX ™ transfection agent (Ambion, Austin, TX) as recommended by the manufacturer. In short, the SiPORT™ NeoFX ™ was diluted in Opti-MEM® Reduced Serum Media (Invitrogen, Carlsbad, CA) and incubated for 10 min at room temperature. miR-155 mimic (Pre-miR™), inhibitor (Anti-miR™), positive control (anti-let-7c), and negative control (Negative control #1) were diluted in Ishikawa media to a final concentration of 30 nM and then combined with the transfection agent and incubated for 10 min at room temperature. Transfection mixtures were added to 6-well plates and overlaid with cell suspensions. Cells were then incubated for 24 hours prior to treatment with peritoneal fluid from control and endometriosis patients, as previously described. Transfections were tested for effectiveness by collecting cells in Qiazol and using a miR-155 primer assay to assess miRNA expression. This transfection process was completed for collection of cells for protein and RNA and a miR-155-5p qPCR assay was used to verify transfection efficiency. RNA was isolated using the miRNeasy Mini Kit following the manufacturer's recommendations. Western blots and

RT-qPCR were used (as previously described) to determine the expression of key downstream targets.

Chromatin Immunoprecipitation (ChIP)

Approximately 70% confluent Ishikawa cells were treated with 1% PF from patients for 48 hours in a DMEM/F12 media containing 1% charcoal-stripped FBS. Proteins were cross-linked to proteins by adding formaldehyde (0.75% by volume) and allowing for a 10-minute incubation at room temperature. Glycine (0.5M) was added and incubated for an additional 10 minutes. Cells were twice rinsed with PBS, collected in 1 ml PBS, pelleted by centrifugation, and lysed in 300 µL of lysis buffer (1% SDS; 5 mM EDTA; 50 mM Tris-HCl, pH 8) plus protease inhibitors (Thermo Scientific). Cell extracts were sonicated on ice 3 x 10 seconds at 15% amplitude using the Sonic Dismembrator Model 500 (Fisher Schientific). Shearing was verified by running chromatin samples on an agarose gel and fragments averaged about 800 kD in size. After isolating 50µl of each sample for INPUT, 100µl per antibody were diluted 1:10 in dilution buffer (16.7 mM Tris-HCl, pH 8; 167 mM NaCl; 1.2 mM EDTA; 0.01% SDS; 1.1% Triton X-100) and rotated overnight at 4°C with 2 µg of non-specific IgG (Santa Cruz) or ChIP-grade anti-Jumonji protein antibody (Cell Signaling, #13594). Antibody-chromatin complexes were collected using 5 µL of magnetic Dynabeads protein A beads (Invitrogen) with rotation at 4°C for 90 min. Using magnetic separation (Life Technologies), beads were washed sequentially with low and high salt wash buffer, 0.25M LiCl wash buffer, and TE buffer. All samples (including INPUT) were incubated at 65°C for four hours with elution buffer containing proteinase K. DNA concentration was determined by NanoDrop 2000 spectrophotometer and analyzed using the Human Polycomb & Trithorax Complexes EpiTect ChIP qPCR Array (Qiagen). This array consisted of primers for genes belonging to the polycomb and trithorax

complexes (core, alternate, and additional compnents), as well as polycomb co-factors such as PHD finger protein 19 (PHF19) and heterochromatin (CBX) proteins. Percent enrichment and further statistical analysis was calculated using an SA Biosciences spreadsheet.

Statistical Analysis

Prism software (GraphPad, Inc., La Jolla, CA) was used for analysis of non-array qPCR data in human tissue and cell culture studies. All values were expressed as mean ± standard error of the mean (SEM). A one-way ANOVA followed by Tukey's post-hoc test was used to detect differences in relative gene expression among treatment groups. Student's t-test was used to compare the means of mRNA and protein expression in various tissues and cell treatments. *P* values less than 0.05 were considered significant.

RESULTS

PRC2 and FOXP3 Expression in Patient Tissues

Protein levels of EZH2, H3K27me3, and FOXP3 in EuNN, EuYY, and EcYY tissues (n=6) are shown in the Western blot in Figure 4.1A. No significant difference was seen between the mean density of endo tissue and control tissue bands (Figure 4.1B). qPCR was used to determine the expression of PRC2 components *SUZ12*, *EED*, and *EZH2*, as well as *FOXP3* (Figure 4.1C). When compared to the control tissues, expression of all four genes was lower in eutopic tissue from endometriosis patients (EuYY). In contrast, ectopic tissues expressed higher levels of *EED* and *EZH2* than control tissues, with a *p*-value of 0.06.

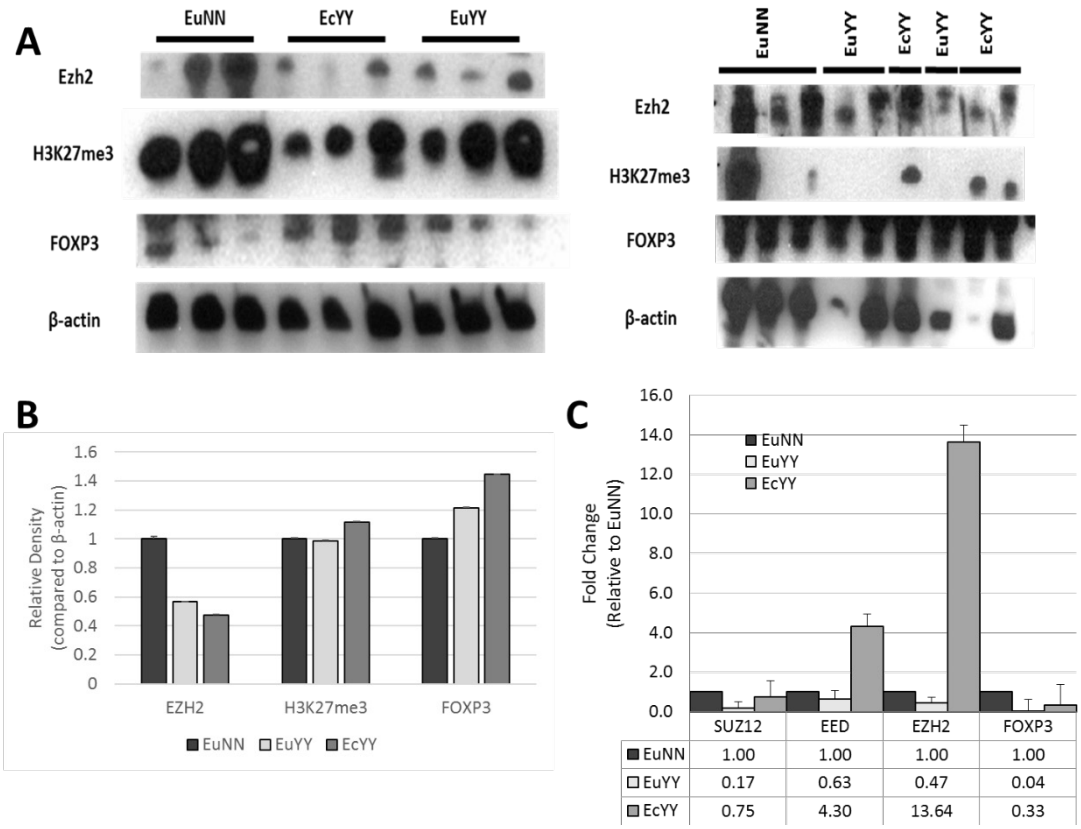

Figure 4.1. Protein and gene expression of key epigenetic mediators in endo and control tissues. A) Western blots of EZH2, H3K27me3, and FOXP3 in control (EuNN), eutopic endo (EuYY), and ectopic endo (EcYY) tissues (n=6). Each blot represents identical sample loading, antibody dilutions, and overall protocol. **B)** Densitometry analysis of Western blots, showing protein expression relative to β-actin; $p>0.05$ **C)** Relative mRNA expression of polycomb repressor complex 2 (PRC2) elements in EuNN (n=3), EuYY (n=7) and EcYY tissues (n=4). In general, these elements were underexpressed in eutopic endo tissues compared to control tissues. There was general overexpression in ectopic endo tissues, especially in *EZH2* (p=0.06).

Expression of Jumonji Protein 2 and Targeting MiRNAs in Patient Tissues

miRNA qPCR assays were used to measure expression of miR-148a, miR-29a, and miR-155, which, among others, target Jumonji protein 2 (Targetscan, IPA). All three miRNAs were overexpressed in endo (eutopic and ectopic) tissues compared to control tissues (Figure 4.2). miR-29a and miR-155 had more pronounced expression in endometriotic lesions than other tissue groups, while miR-148a expression was greatest in EuYY tissues. Compared to control

tissues, Jumonji protein 2 was significantly downregulated in eutopic endometrium from endometriosis patients (p=0.004).

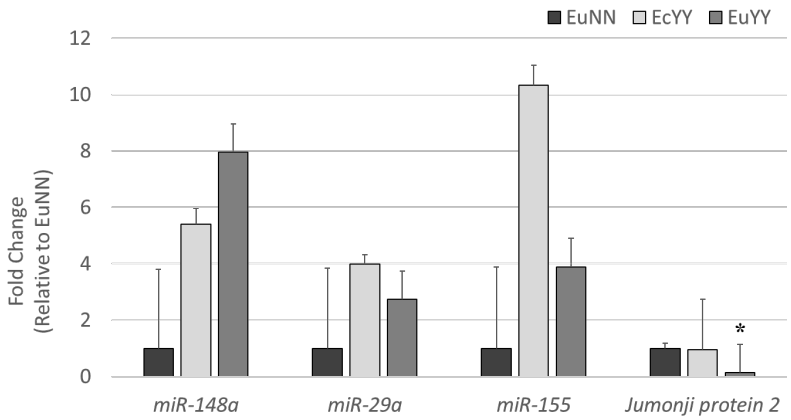

Figure 4.2. Expression of Jumonji protein 2 and miRNAs that target Jumonji protein 2 in patient tissue samples. Compared to control tissues (n=3), expression of miR-148a, miR-29a, and miR-155 were all higher in endo tissues (both eutopic and ectopic, n=3). Jumonji protein 2 was significantly underexpressed in eutopic tissues from endo patients when compared to control tissues (p=0.004).

Epigenetic Expression in an Endo Cell Model

Protein and mRNA expression of epigenetic regulators were measured in endometrial cells treated with peritoneal fluid from women with and without endometriosis and pain (Figure 4.3). Cells treated with endo PF (YN-PF and YY-PF) had increased Jumonji protein 2, *EZH2*, and *FOXP3* mRNA expression compared to media control. NN-PF had no such induction. The corresponding protein expression was similar among the PF treatments. Despite the significant decrease in EZH2 upon YN-PF treatment compared to media control (0.66-fold change, p=0.032), there was no such discernable difference when it was compared to NN-PF treatment. FOXP3 protein expression was significantly lower (0.763-fold) in YY-PF treated cells when compared to NN-PF treated cells. The double band seen in Figure 4.3C could be explained by post-translational modifications to its regulatory elements. To test for this, calf intestinal phosphatase (CIP) could be added to the samples, resulting in fused bands or elimination of the band representing phosphorylation. Trimethylation of H3K27 was also significantly less

prevalent in YY-PF treated cells (0.679-fold). Based on the molecular weight of the modification (about 17 kDa), the top bands were measured for densitometry.

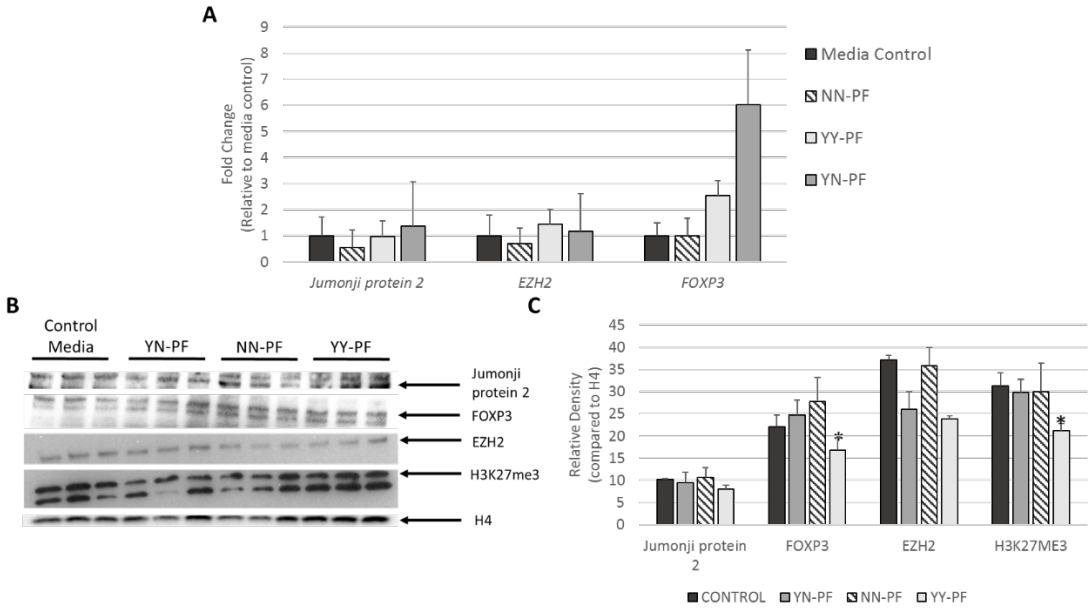

Figure 4.3. mRNA and protein expression of epigenetic mediators in an endo cell model. A) mRNA expression of *Jumonji protein 2*, *EZH2*, and *FOXP3* in cells treated with NN-PF (n=6), YY-PF (n=5), and YN-PF (n=3), relative to expression in a media control ($p>0.05$). **B)** Western blots for Jumonji protein 2, FOXP3, EZH2, and H3K27me3 in PF-treated cells. Arrows indicate the appropriate row of bands. **C)** Densitometric analysis of Western blots. Relative protein expression of Jumonji protein 2, EZH2, and FOXP3 in PF-treated cells was calculated in relation to a media control. FOXP3 expression was 0.763-fold lower in YY-PF than in control media; H3K27me3 levels were 0.679-fold lower in YY-PF than in media control; $p<0.05$

miR-155 Regulates PRC2 Complex

The expression of Jumonji protein and the PRC2 complex was determined in endometrial cells transfected with a miR-155 mimic or antagonist (Figure 4.4). The miR-155 mimic had minimal effect on Jumonji protein expression in PF-treated cells ($p>0.05$), but seemed to increase FOXP3 expression in cells treated with control PF. The miR-155 inhibitor significantly decreased Jumonji protein expression in cells treated with YY-PF ($p=0.0172$).

Overexpression of miR-155 resulted in significantly higher Jumonji protein expression in endo PF-treated cells compared to control PF-treated cells (YY-PF $p = 0.005$, YN-PF $p = 0.002$)

(Figure 4.5A). FOXP3 expression was significantly lower in all PF-treated cells than media control cells transfected with miR-155 mimic (NN-PF $p = 0.0112$, YY-PF $p = 0.0002$, YN-PF $p = 0.0005$). Inhibition of miR-155 resulted in significantly higher Jumonji protein expression in YN-PF treated cells compared to NN-PF treated cells ($p<0.001$) (Figure 4.5B). EZH2 expression was lower than control media in NN-PF and YY-PF treated cells but higher in YN-PF treated cells (NN-PF $p = 0.053$, YY-PF $p = 0.006$, YN-PF $p = 0.026$). Compared to the media control transfected with miR-155 antagonist, the expression of FOXP3 was significantly higher in all PF-treated cells (NN-PF $p<0.001$, YY-PF, $p = 0.006$, YN-PF $p<0.001$). Trimethylation of H3K27 was less prevalent in endo PF-treated cells compared to NN-PF treated cells (YY-PF $p = 0.025$, YN-PF $p<0.001$).

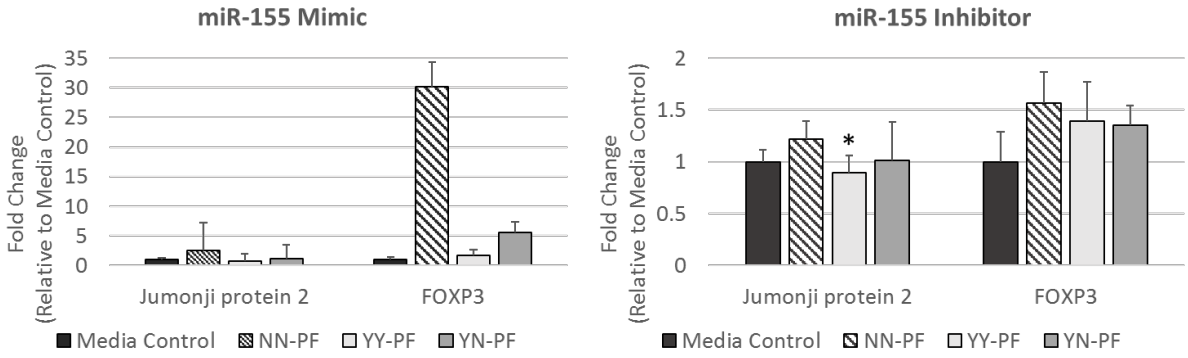

Figure 4.4. Key mRNA levels in cells transfected with a miR-155 mimic and antagonist. Transfection with a miR-155 mimic had little effect on *Jumonji protein 2* expression in PF-treated cells (p>0.05), but seemed to increase *FOXP3* expression in cells treated with control PF. Compared to control media, the miR-155 antagonist significantly decreased *Jumonji protein 2* expression in cells treated with YY-PF (*p=0.017)

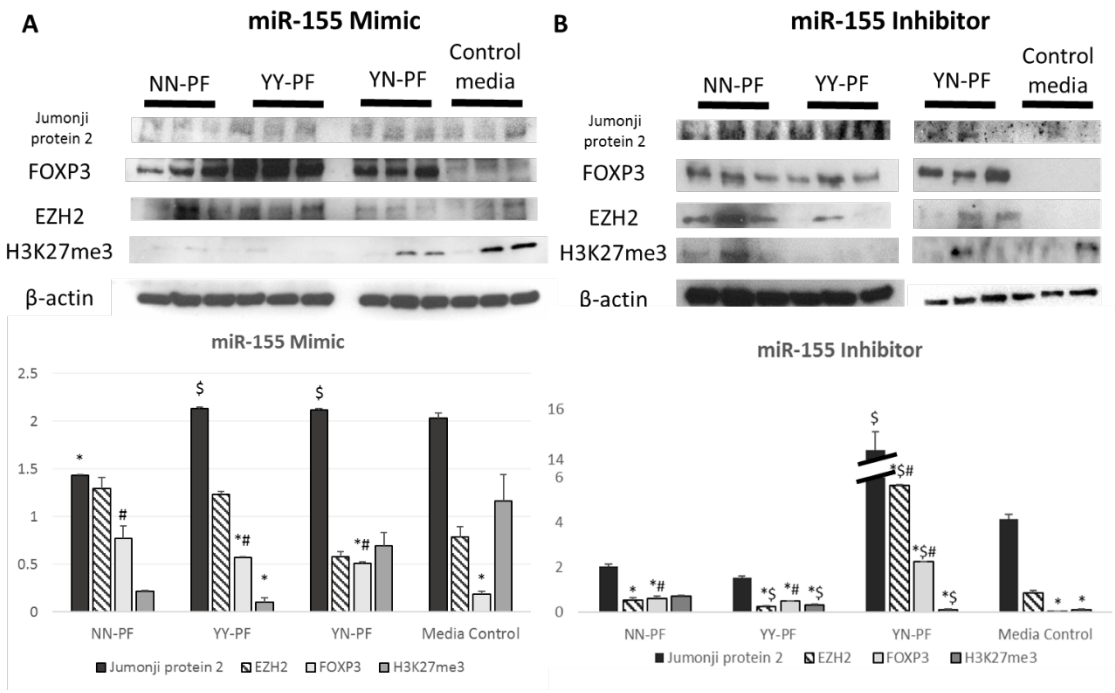

Figure 4.5. Expression of key proteins in cells transfected with miR-155 mimic and antagonist. A) Transfection with a miR-155 mimic resulted in significantly higher Jumonji protein 2 expression in endo PF-treated cells (n=3) compared to control PF-treated cells (n=3)(YY-PF $p = 0.005$, YN-PF $p = 0.002$) and lower FOXP3 expression in all PF-treated cells compared to media control cells transfected with miR-155 mimic (NN-PF $p = 0.0112$, YY-PF $p = 0.0002$, YN-PF $p = 0.0005$. **B)** Transfection with a miR-155 inhibitor resulted in significantly higher Jumonji protein 2 expression in YN-PF treated cells compared to NN-PF treated cells ($p<0.001$). EZH2 expression was lower than control media in NN-PF and YY-PF treated cells but higher in YN-PF treated cells (NN-PF $p = 0.053$, YY-PF $p = 0.006$, YN-PF $p = 0.026$). Compared to the media control transfected with miR-155 antagonist, the expression of FOXP3 was significantly higher in all PF-treated cells (NN-PF $p<0.001$, YY-PF, $p = 0.006$, YN-PF $p<0.001$). H3K27me3 was less prevalent in endo PF-treated cells compared to NN-PF treated cells (YY-PF $p = 0.025$, YN-PF $p<0.001$).
*Significant difference ($p<0.05$) in mean compared to control media, $^{\$}$Significant difference ($p<0.05$) in mean compared to NN-PF, $^{\#}$Significant difference ($p<0.05$) in mean compared to control media with mimic/inhibitor

Epigenetic Regulation of FOXP3

Global DNA methylation array to assess promoter methylation patterns showed changes in genes involved in Inflammation and autoimmunity. The heat map in Figure 4.6 presents a range (from 0 to 100) of "M", the fraction of input genomic DNA containing 2+ methylated CpG sites in the targeted region of a gene. Based on the fold changes ($p>0.05$ in all instances), the

following five genes were identified as being impacted by DNA methylation: Forkhead box protein J1 (*FOXJ1*), MHC class I polypeptide-related sequence B (*MICB*), forkhead box P3 (*FOXP3*), Cluster of Differentiation 8a (*CD8A*), and Lymphotoxin Beta (*LTB*). All genes, except *CD8A*, had an increased methylation pattern in cells treated with endo PF (YN and YY) compared to NN-PF and the media control. *FOXP3* M values were 56.67% in YY-PF treated cells, 25.54% in NN-PF treated cells, 33.73% in YN-PF treated cells, and 0.23% in control media. Bisulfite sequencing will be used in the future to better understand the methylation patterns of sample DNA.

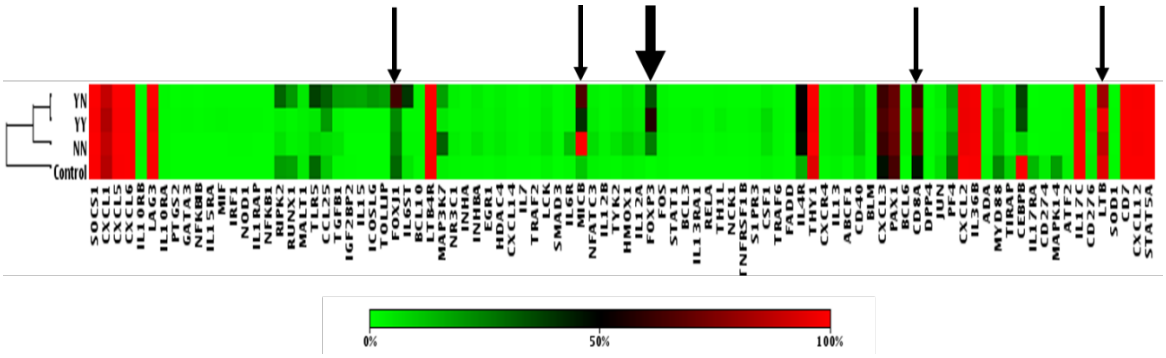

Figure 4.6. Methylation patterns of inflammatory genes in PF-treated cells. Heat map showing DNA methylation trends in PF-treated cells on promoters of genes associated with autoimmunity and inflammation. Treatment groups with green shades have lower methylation fractions than those with red shades. Of interest is *FOXP3*, which has been deemed a tumor suppressor gene. YY-PF: M = 56.67%; YN-PF: M = 33.73%; NN-PF: M = 25.54%; Control media: M = 0.23%.

Jumonji Protein Regulates PRC2 Target Genes

To determine the epigenomic targets of Jumonji protein 2 we performed ChIP using Jumonji Protein 2 antibody followed by promoter array of genes associated with polycomb and trithorax complexes in cells treated with PF. Generally, there was less enrichment of the genes (*BAP1, EED, EZH1, EZH2, PCGF1, PCGF2, PCGF5, PHF1, PHF19*) when cells were treated with endo PF. Figure 4.7 provides a graphical representation of genes with notable differences in YY-PF and YN-PF, when compared to NN-PF. The enrichment of EZH2 by Jumonji protein 2

was lower in endo PF-treated cells compared to NN-PF treated cells. EZH1, a polycomb enzyme which is responsible for mono-, di-, or tri-methylation of H3K27, showed greater enrichment by Jumonji protein 2 in YY-PF treated cells than NN-PF treated cells. PCGF represents the polycomb group ring finger genes, which had similar fold change values in both endo treatment groups. For all data, $p>0.05$, likely due in part to small sample size.

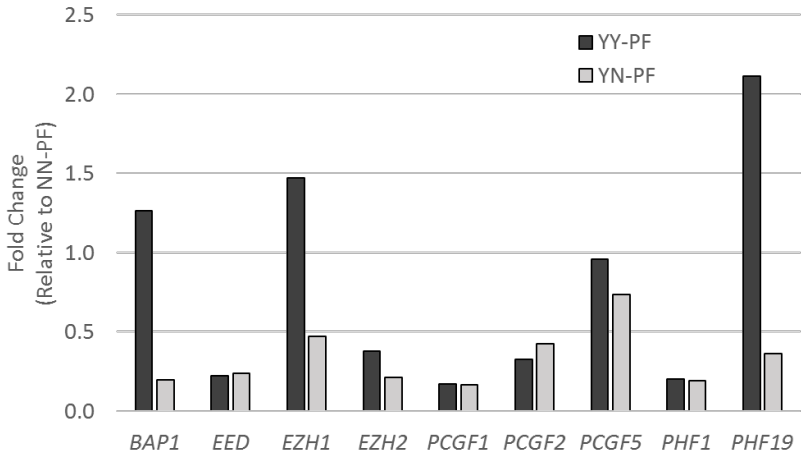

Figure 4.7. **Epigenetic crosstalk in PF-treated cells.** Chromatin Immunoprecipitation to analyze interactions between Jumonji protein 2 and genes associated with the polycomb and trithorax complexes, normalized to IgG. Fold change values represent the ratio of enrichment/binding of Jumonji protein 2 to various genes in endo PF-treated cells (n=3) to enrichment in control PF-treated cells (n=3). Generally, increased enrichment was seen on genes in the presence of YY-PF compared to YN-PF. However, fold change indicates that both endo conditions resulted in lower enrichment compared to control PF. $p>0.05$. BAP1: BRCA1 associated protein-1; EED: Embryonic ectoderm development; EZH1: Enhancer of zeste homolog 1; EZH2: Enhancer of zeste homolog 2; PCGF1: Polycomb group ring finger 1; PCGF2: Polycomb group ring finger 2; PCGF5: Polycomb group ring finger 5; PHF1: PHD finger protein 1; PHF19: PHD finger protein 19

DISCUSSION

Our laboratory has been working to identify underlying mechanisms of pain experienced by endometriosis patients (K. Ray et al., 2015; K. L. Ray et al., 2014; Rong et al., 2002; Santanam et al., 2013). This study stemmed from investigations into the miRNA profile of endometriosis tissues and PF. Nineteen percent of differentially expressed miRNAs in endo

tissues targeted Jumonji protein 2. Despite the global downregulation seen in the micronome of endometriotic tissues (Wright, 2017), miRNAs that targeted Jumonji protein 2 were highly expressed in the eutopic tissues of endometriosis patients with pain. The overexpression of miR-148a, miR-29a (Wright, 2017), and miR-155 in endo tissues (Figure 4.2) seem to support this theory. Although little to no difference in PRC2 protein expression was seen among tissues, there was a noticeable trend in overexpression of corresponding genes in ectopic tissues from endometriosis patients, particularly in *EZH2* (p=0.06). This correlates with the findings of Colon-Caraballo and colleagues (Colon-Caraballo 2015) and supports the characterization of EZH2 as a contributor to transcriptional repression and progression of the disease. FOXP3 was present in lesser amounts in endo tissues compared to control tissues. Based on the role of FOXP3 as a tumor suppressor and the tendency for inflammation to repress its expression (Gao et al., 2015), these results are not surprising.

Although miR-155 was not originally identified based on the micronome array ($p>0.05$), its relationship with Jumonji protein 2 has recently drawn the attention of researchers in the field of inflammatory disease (Escobar et al., 2014; Palma et al., 2014). miR-155 seems to play a key intermediary target that regulates the crosstalk between Jumonji protein 2 and PRC2. Hence miR-155 is a potential therapeutic target. In this study, we explored the role of miR-155 in endometriosis by transfecting PF treated cells with a miR-155 mimic or antagonist. Despite the knowledge that FOXP3 targets miR-155 (Kohlhaas et al., 2009), overexpression of miR-155 in PF-treated cells resulted in little to no difference in FOXP3 expression among the three treatment groups. However, blocking miR-155 resulted in significant overexpression of FOXP3 in YN-PF treated cells. The effect of altering miR-155 on Jumonji protein 2 was also interesting. Compared to control PF-treated cells, cells treated with endo PF overexpressed Jumonji protein 2 when

transfected with the miR-155 mimic. When transfected with the miR-155 inhibitor, only cells treated with YN-PF overexpressed Jumonji protein 2 compared to control PF treatments. These results were unexpected and suggest that the miRNA regulation of FOXP3 and Jumonji protein 2 is not sufficient to alter expression. Other transcription factors and/or epigenetic mediators could play a role in aberrant expression in endometriosis. Knocking down miR-155 provided the most insight into the mechanism. While the presence of pain seemed to contribute to the downstream repression of EZH2, the repression of the histone modification was seen in cells treated with both endo PFs in comparison to NN-PF treated cells, suggesting that this interaction may be related to the presence of endometriosis independent of the pain symptom.

Methylation of the FOXP3 promoter could be partly responsible based on the trend of increased methylation in cells treated with PF from endo patients, particularly those reporting pain. *FOXP3* expression in endo PF-treated cells trended lower than that of cells treated with control PF and protein expression varied very little among the treatment groups. EZH2 expression was significantly lower in cells treated with YN-PF than in the media control but no difference was seen *among* PF treatment groups. The benefit of studying these epigenetic mediators in tissues and treated cells gave us the ability to compare short-term and long-term effects of peritoneal fluid on endometrial cells. This difference is likely to contribute to explaining the disparities in results.

ChIP-qPCR was used to better understand the role that Jumonji protein 2 plays in endometriosis-associated pain. By observing how it binds to regulatory elements of various genes, we gained a sense of how the mechanisms described above differ between PF from patients with pain to those without. The preliminary data presented in Figure 4.7 suggest that the Jumonji protein 2 interaction with EZH2 may not be as strong in a “painful” situation as it is

with EZH1, which can also methylate H3K27 to contribute to transcriptional repression. Although it is typically associated with active domains, EZH1 can actually achieve repressive results similar to EZH2 via additional histone modifications (Mochizuki-Kashio et al., 2015; X. Shen et al., 2008; Son, Shen, Margueron, & Reinberg, 2013). It is interesting to note that, in general, binding of Jumonji protein 2 to these genes was less likely to occur in cells exposed to endo PF compared to control PF. One exception was *PHF19*, where enrichment appeared to be greater in cells treated with YY-PF compared to both NN-PF and YN-PF ($p>0.05$). *PHF19* has the ability to bind H3K36me3, which allows it to act as a recruiter for the PCR2 complex (Brien et al., 2012; S. Qin et al., 2013), suggesting that another mechanism may be at play in transcriptional repression. PHF19 has also been deemed a role player in the switch from proliferative to invasive states in melanoma cells (Ghislin, Deshayes, Middendorp, Boggetto, & Alcaide-Loridan, 2012). Additional ChIP experiments are needed to confirm the enhanced binding of Jumonji protein 2 to this gene and ChIP-re-ChIP studies would investigate the activity of the Jumonji protein by encompassing trimethylation of H3K27.

The findings presented here, as summarized in Figure 4.8, provide potential mechanisms for inflammatory pain and proliferation in endometriosis patients. This opens the door for novel therapies such as EZH2 inhibitors and miRNA mimics/antagonists. Future studies will test such therapies (e.g. GSK126 and sulforaphane, established as anti-inflammatory agents) in cell and animal models of endometriosis. Although histone demethylases are thought to be ineffective against Jumonji protein 2 due to its lack of true demethylase activity, additional investigations into the role of Jumonji protein 2 in endometriosis could uncover alternative options to therapeutically regulate it.

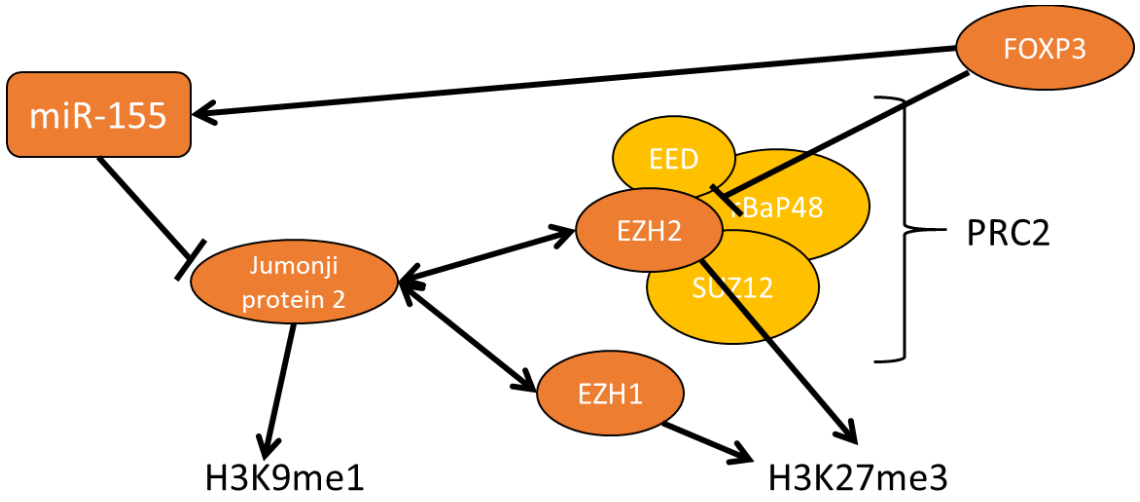

Figure 4.8. Proposed schematic of the epigenetic mechanism contributing to the pain and progression of endometriosis. Arrows indicate activation or general targeting while "T" bars indicate inhibition.

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GENE NAME	PRIMER TYPE	SEQUENCE
EED	sense	5′- CATTGGGCAATCAAGTTGGCA -3′
	antisense	5′- ACAAGTGTGGAGAAAAAGCCTG -3′
SUZ12	sense	5′- GTTACCGGTGAAGAAGCCGA -3
	antisense	5′- TTGGCTTCTCAAAGGCCTGG -3′
EZH2	sense	5′- AAGGAGTTTGCTGCTGCTCT -3′
	antisense	5′- ATTAATGGTGGGGGTGCTGG -3′
Jumonji protein 2	sense	5′- CTGCAGCACAAACGTGACTT -3′
	antisense	5′- CATCAGCGAAACGTGAAGGTC -3′
FOXP3	sense	5′- ACTGGGGTCTTCTCCCTCAA -3′
	antisense	5′- GGGATTTGGGAAGGTGCAGA -3′

Supplementary Table 4.1. Primer sequences for RT-qPCR analysis of epigenetic gene expression in patient tissues and PF-treated cells. Primers were designed using NCBI GenBank and ordered from Invitrogen.

CHAPTER 5

SUMMARY AND CONCLUSION

The goal of this thesis was to expose the underlying mechanisms of chronic pelvic pain suffered by many endometriosis patients. Our laboratory has a strong history of investigating the role of oxidative stress and oxidatively-modified lipoproteins in endometriosis-associated inflammation (Murphy, Santanam, Morales, et al., 1998; Murphy, Santanam, & Parthasarathy, 1998; Parthasarathy et al., 2010; Rong et al., 2002; Santanam et al., 2013; Santanam, Murphy, et al., 2002; Shanti et al., 1999). We know that ox-LDLs present in the peritoneal fluid and tissues of endometriosis are partially responsible for the high inflammatory state and progression of the disease. The work presented here further explored *how* these ox-LDLs accomplish this as well as the role of nociceptive pain associated with the disorder.

As was mentioned in the introduction of this thesis, there are multiple types of pain associated with endometriosis, so addressing just one therapeutically is unlikely to provide sufficient relief. One alternative is to consider the interplay of inflammatory and nociceptive pain. There is evidence that nerve fibers which innervate ectopic and eutopic tissues of endometriosis patients terminate in nociceptors that are sensitive to inflammatory markers (Berbic & Fraser, 2011). We hypothesized that the ox-LDLs present in the peritoneal fluid of patients are responsible for the overexpression of several such inflammatory and nociceptive markers (e.g. IL6, OPRM1, and CX3CR1) compared to the PF from control patients. These prostaglandin-like molecules exacerbated the genetic pain condition as well as the nociceptive response in a mouse model. While treatment with NSAIDs and antioxidants addressed these conditions and improved responses in the animal model, understanding the mechanism of action of ox-LDLs is crucial.

There are numerous parallels between the progression of endometriosis and cancer, so it comes as no surprise that there are similar mechanisms behind them. One example is the global downregulation of human miRNAs in patients of both diseases. In order to take advantage of this trend, it is necessary to study any downstream targets of these small RNAs. We found that, of 1000+ miRNAs in the human micronome, relatively few were aberrantly expressed only in cells treated with PF from endometriosis patients *and* those treated with ox-LDLs (Figure 3.2). Even fewer had validated target genes associated with pain (Figure 3.5). The let-7 family of miRNAs, particularly, let-7i and let-7g, were prime examples of potential ox-LDL regulators in both tissues and endo cell models. Figure 5.1 summarizes how let-7 and other miRNAs accomplish inflammatory and/or nociceptive responses in the peritoneal cavity of endometriosis patients.

The final study described here led us to believe that endometriosis-associated pain, in addition to being associated with the afore-mentioned mechanisms, is epigenetic in nature. FOXP3 is a known tumor suppressor and has become a novel subject of investigation in cancer research. Its role as a transcription factor in immune response means that any alterations to its expression could have consequential effects. In a cell model with PF from endometriosis patients, *FOXP3*'s promoter was hypermethylated compared to cells treated with PF from control patients, particularly when the PF was from patients with pain. This methylation and subsequent downregulation of FOXP3 could contribute to the overexpression of proinflammatory miR-155. Assuming that the proposed relationship between miR-155 and PRC2 is correct, it is plausible that the miRNA works to increase EZH2 expression and even activity via Jumonji protein 2.

The studies completed as part of this dissertation have successfully unveiled some of the mechanisms behind endometriosis-associated pain. Studying the role of inflammation and

nociception in both tissues and PF-treated cells allowed us to observe short- and long- term effects of the complex milieu that is peritoneal fluid. Key limitations of these studies include sample size, particularly for specimens from endometriosis patients without pain, as most endometriosis patients were symptomatic. Nevertheless, we are confident in the findings presented here. There is clear evidence for the role of PF-based ox-LDLs in endometriosis-associated pain as well as some downstream targets of these molecules. The micronome and epigenetic profile of endometriosis patients with pain could also hold a key to effective treatment. In the future, it would be ideal to consider antioxidant, miRNA, and/or EZH2 inhibitor therapy in conjunction with NSAIDs. Further studies could investigate the effectiveness of these options in an animal model. Certainly there is a lot of promise in the findings presented here.

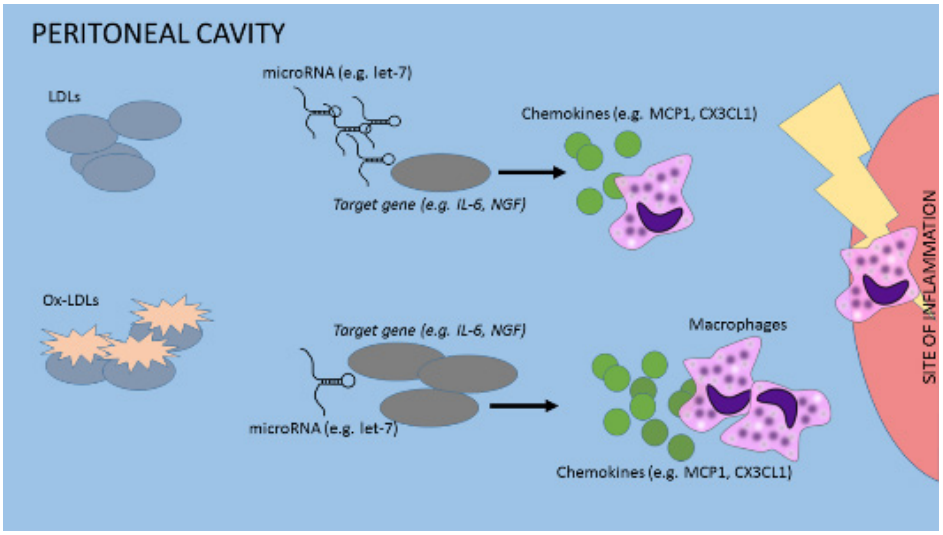

Figure 5.1: Overview of the ox-LDL-triggered mechanism of miRNAs in the peritoneal cavity of endometriosis patients. In the presence of oxidized LDLs, a downregulation of miRNAs leads to an overexpression of inflammatory and nociceptive genes. This triggers the release of additional chemokines and exacerbation of the inflammatory state of the cavity.

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APPENDIX A

IRB APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity
Institutional Review Board

Wednesday, May 14, 2008

Nalini Santanam, Ph.D.,MPH
Pharmacology
Byrd Bio Basic Science Ctr.
1700 3rd Ave., #412 BBSC
Huntington, WV. 25755

RE: IRB Study # 9074 **At:** Marshall IRB 1

Dear Dr. Santanam:

Protocol Title:
Pain Molecules in the Peritoneal Fluid of Women with Endometriosis

Expiration Date:	5/13/2009	
Our Internal #:	4839	
Type of Change:	Annual Report	Approved
Expedited ?:	☐	
Date of Change:	5/14/2008	
Date Received:	5/14/2008	
On Meeting Date:	5/14/2008	
Description:	The above study and informed consent were unanimously approved at today's full board convened meeting of the Marshall University IRB #1 for the period of 12 months. The approval will expire 05/13/09. Continuing review materials must be submitted no later than the deadline for the April 8, 2009 full board IRB agenda. The purpose of the study is to identify the source of pain and to detect molecules that may be inducing pain the peritoneal fluid of women with endometriosis.	

The co-investigators are Drs. Brenda Dawley and Andreia M. Acuna (MU/CHH).

Respectfully yours,

Henry K. Driscoll, M.D.
IRB Chairperson

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APPENDIX B

IACUC APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Animal Resource Facility

DATE:	March 4, 2015
TO:	Nalini Santanam, PhD, MPH
FROM:	Marshall University IACUC
IACUC #:	485
PROJECT TITLE:	[397498-5] Pain Inducing molecules in the peritoneal fluid of women with endometriosi- Rat Model
SUBMISSION TYPE:	New Project
ACTION:	APPROVED
APPROVAL DATE:	March 4, 2015
EXPIRATION DATE:	March 3, 2018
REVIEW TYPE:	Full Committee Review

Thank you for your submission of New Project materials for this research project. The Marshall University IACUC has APPROVED your submission. All research must be conducted in accordance with this approved submission.

This submission has received Full Committee Review.

Please note that any revision to previously approved materials must be approved by this committee prior to initiation. Please use the appropriate revision forms for this procedure.

Please report all NON-COMPLIANCE issues regarding this project to this committee.

This project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Monica Valentovic at (304) 696-7332 or valentov@marshall.edu. Please include your project title and reference number in all correspondence with this committee.

Monica A. Valentovic, Ph.D.
Chairperson, IACUC

Kristeena Ray Wright
kristeena.ray@gmail.com
ray137@marshall.edu
1329 Washington Blvd., Huntington, WV 25701
(804) 852-7506

EDUCATION

Ph.D. Student in Biomedical Sciences, Focus in Toxicology and Environmental Health, Marshall University, Huntington, WV
Relevant Coursework: Cell Biology/Biochemistry, Protein Biotechnology, Animal Physiology, Advanced Molecular Genetics, Medical Pharmacology, Toxicology
B.S. in Biomedical Engineering, December 2009, Duke University, Durham, NC

RESEARCH EXPERIENCE

Graduate Student, Fall 2011 to present
Marshall University, Dr. Nalini Santanam
Assessing the role of oxidative stress and epigenetics in the etiology of endometriosis and chronic pain using the following techniques and reagents:

- Plantar test (Hargreaves' Method) to assess pain in animal models
- Chromatin immunoprecipitation (ChIP)
- Reverse transcription
- Real-time polymerase chain reaction (PCR) to determine presence and expression of cytokines and nociceptive/inflammatory genes in human peritoneal fluid and endometrial tissue
- RNAi cell transfection
- Bacterial cell culture and transformation
- Sterile culture of EM42 and Ishikawa human endometrial cell lines
- Sterile cell culture of primary endometrial cells
- SDS-PAGE and Western blotting
- Bioluminescence imaging
- RNA and DNA isolation
- Protein quantification by Lowry method

Capstone Student Mentor, Summer 2015**;** Fall 2014; Summer and Fall 2013, Marshall University

- Recruited undergraduate students to work on an endometriosis-related project under my direction
- Developed a research plan that led to a comprehensive presentation
- Overseeing development of report that outlines experimental design and results

Summer Student Mentor, Summer 2015, Summer 2012

- Assisted in developing and overseeing projects of students in the NIH-funded WV-INBRE program and SRIMS program

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PUBLICATIONS

Redox Regulation of microRNAs in endometriosis-associated pain
Kristeena Ray Wright, Brenda Mitchell, Nalini Santanam. *Redox Biology* 12 (2017): 956-966.
Mitochondrial redox status as target for cardiovascular disease.
James Walters, Deborah Amos, Kristeena Ray, Nalini Santanam. *Current Opinion in Pharmacology* April 2016; 27:50-55.
Oxidation Sensitive Nociception Involved in Endometriosis Associated Pain
Ray K, Fahrmann J, Mitchell B, Paul D, King H, Crain C, Cook C, Golovko M, Brose S, Golovko S, Santanam N. *Pain* Mar 2015;156(3):528-39.

Power over Pain: A brief review of current and novel interventions for endometriosis-associated pain
Kristeena Ray, Brenda Mitchell, Nalini Santanam. *Journal of Endometriosis and Pelvic Pain Disorders* Dec 2014; 6(4):163-173.

TECHNICAL SKILLS

Additional Laboratory: Filtration and Filling, Micropipetting, Centrifugation, Spectrophotometry and Absorbency analysis, Moisture analysis (NIR, Karl Fischer assay, Lighthouse), Lyophilization
Operating Systems: Microsoft Windows, iOS
Software: Microsoft Office Suite (Word, Excel, PowerPoint, Outlook), EndNote, Adobe Photoshop, Ingenuity Pathway Analysis, Biorad ImageLab, GraphPad Prism, Laboratory Information Management System

TEACHING EXPERIENCE

Guest lecturer, BMS 600 – Biochemistry/Cell Biology, Marshall University, Fall 2014, 2015, and 2016

- *Taught Epigenetics and ncRNA lectures to first year research and Medical Sciences students and assessed knowledge with exam questions*
- *Led discussion section about relevant historical publication*

Guest speaker, Women's Studies 101 course, Marshall University
Invited to be annual guest speaker discussing women in science and research, endometriosis

SCHOLARSHIPS and GRANTS

2014 American Society for Cell Biology (ASCB) Local Meetings Grant

- Primary contributor to an American Society for Cell Biology grant application for a local conference for graduate students in the Appalachian region
- Chair of committee for 2014 Appalachian Regional Cell Conference (ARCC)
    - Campaigned to have meeting on a college campus for the first time
    - Liaison between ASCB and ARCC committee
    - Identified and booked Keynote speaker, Dr. Beth Weaver of University of Wisconsin
    - Organized space and catering reservations

Chancellor's Scholars Program, Marshall University

- Member of inaugural class of program rewarding and supporting minority doctoral students
- Full scholarship covers tuition for remainder of Ph.D. process

AFFILIATIONS

American Society for Biochemistry and Molecular Biology, 2012-present

- Active participant in 50 State Challenge to promote Science Policy
    - Hosted a Science Policy seminar for biomedical sciences graduate students
    - Presented idea to graduate program for a congressional meeting on campus
    - Subject of December Policy Blotter Spotlight on ASBMB Advocacy website
- 2015 ASBMB Capitol Hill Day Participant, June 2015
- Awarded 2013 travel grant to Experimental Biology conference in Boston
- Subject of article published by *ASBMB Today* editor and released to national media

American Society for Cell Biology, 2014-2016
Graduate Student Organization, President - 2013-2014, Marshall University BMS program
Women in Medicine and Science, Executive Council member, Marshall University
Southern Regional Educational Board, Doctoral Awardee

- Attend annual leadership Compact for minority doctoral students in the southern region of the US

National Society of Black Engineers

HONORS and AWARDS

Keynote Speaker, 2015 Intercultural Students' Weekend, hosted by Marshall University Office of Intercultural Affairs

Keynote Speaker, 2015 Marshall University Freshmen Convocation

Best Overall Performance by a Graduate Student, August 2015

Top-performing Biomedical Sciences research candidate awarded stipend to attend an international research conference

2015 ASBMB Capitol Hill Day Participant, June 2015

Chosen as one of twenty participants granted the chance to help promote scientific research by directly interacting with government officials in Washington, DC

2015 Marshall University Women of Color Award

Awarded to minority female students that show unique dedication to service in the university and local communities with a focus on improving diversity

WCE 2014 Rodolphe Maheux Travel Grant

Awarded to top seven abstracts submitted to present at the World Congress on Endometriosis in Brazil in April 2014

2013 Best. Decision. Ever. Campaign, Marshall University

Student representative for campaign commercial aimed at recruiting for Marshall students
Story and research featured in Alumni Foundation appeal to university alumni

2013 ASBMB Graduate Minority Travel Award, April 2013

Received travel funding for presentation of 'Epigenetic markers in patients with endometriosis'

2012 Appalachian Regional Cell Conference, October 2012, Charleston, WV

Awarded Best Poster in Group at 2012 for presentation on endometriosis-associated pain

ASBMB press release, April 2013

Featured in article 'Pain, epigenetics and endometriosis' by ASBMB Today editor Angela Hopp

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ABSTRACTS, CONFERENCES, and PRESENTATIONS (Summary)

13th World Congress on Endometriosis, May 2017, Vancouver, BC

Selected to give an oral presentation on the ox-LDL regulation of microRNAs in endometriosis

Marshall University School of Medicine Research Day, 2013-2017, Huntington, WV

Chosen for oral presentation and awarded top oral presentation in category in 2015

2015 Appalachian Regional Cell Conference, November 2015, Huntington, WV

Served as Marshall University representative to the conference planning committee

2015 American Society for Reproductive Medicine Annual Meeting, October 2015, Baltimore, MD

'The epigenetic role of Ezh2-FoxP3 crosstalk in endometriosis and its associated pain' abstract selected for poster presentation

12th World Congress on Endometriosis, April 2014, São Paolo, Brazil

Selected to give an oral presentation on the Novel mechanisms of nociception in animal and cell models of endometriosis

Selected to present a poster presentation at the World Congress on Endometriosis

2013 Experimental Biology Conference, April 2013, Boston, MA

Awarded ASBMB Graduate Minority Travel Award for presentation of Epigenetic markers in patients with endometriosis

2012 Appalachian Regional Cell Conference, October 2012, Charleston, WV

Awarded Best Poster in Group at 2012 for presentation on endometriosis-associated pain