REGULATION OF MICRORNA-21 AND ITS ROLE IN FIBROSIS IN THE PATHOPHYSIOLOGY OF ENDOMETRIOSIS

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ABSTRACT

Endometriosis is one of the most common causes of chronic pelvic pain and infertility that affects 10% of women of reproductive age. A delay of up to 9 years is estimated between the onset of symptoms and the diagnosis of endometriosis. Endometriosis is currently defined as the presence of endometrial epithelial and stromal cells at ectopic sites, however, advances in the research of endometriosis has some authors believing that endometriosis should be re-defined as “a fibrotic condition in which endometrial stroma and epithelium can be identified.” There are several theories on the etiology of the disease, but the origin of endometriosis remains unclear. MicroRNAs (miRNAs) are naturally occurring posttranscriptional regulatory molecules that potentially play a role in endometriotic lesion development. The presence of endometriotic lesions can alter miRNAs expression in both the eutopic endometrium and ectopic lesions. There is evidence that suggests that miRNAs, including miR-21, participate in the fibrotic process in different organs including the heart, kidney, liver and lungs. This dissertation has aimed to understand the role of miR-21, which is upregulated in endometriosis, and the mechanisms that can contribute to the development of fibrosis and lesion development in endometriosis. The studies have been focused on the mechanisms by which IL-6 regulates miR-21 and how this miRNA contributes to TGF-β signaling pathway by blocking Smad7, promoting fibrosis and lesion development. These studies collectively showed that inflammation and fibrosis are present at a very early stage of endometriosis. The inflammatory environment in the peritoneal cavity of women with endometriosis, which includes the cytokine IL-6, can regulate the expression of miR-21 *in vitro* and *in vivo*. The upregulation of miR-21 was correlated with the development of fibrosis during

lesion progression of endometriosis, but other components may be also implicated during this process may contribute to the development of fibrosis. These studies also revealed that B cells could play an important role, however their function remains unknown and should be further investigated in the future. Future work should aim to understand the mechanisms that drive miR-21 to regulate fibrosis in endometriosis and the potential therapeutic mechanisms to treat the disease. In addition to this, further studies should also be focused on the role of B cells that would help to explain their function during the development of endometriosis and could potentially serve as suitable candidates for new therapeutic strategies. This could open up different and less invasive approaches for the treatment of women with endometriosis.

This work is dedicated to all those women affected by endometriosis and all of those who supported directly or indirectly women with this disease.

“No puede impedirse el viento, pero hay que saber construir molinos”
Miguel de Cervantes Saavedra
-Don Quijote de la Mancha-

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CHAPTER 1. INTRODUCTION TO ENDOMETRIOSIS

1.1 Definition and an Introduction of the Pathophysiology of Endometriosis

Endometriosis is a benign, inflammatory, fibrotic and estrogen dependent gynecological disorder characterized by endometrial-like tissue outside the uterus, mainly on the pelvic peritoneum, ovaries, and rectovaginal septum. In rare cases, this endometrial-like tissue can be found in uncommon areas such as the pericardium, pleura, diaphragm or even the brain[1-4]. It affects approximately 5-10% of women of reproductive age, and it is associated with pelvic pain and infertility[2, 5]. Endometriosis is a common condition with a profound negative impact on women's lives during their fertile years. Over their lifetime, women with this disorder confront challenges that affect their quality of life, participation in daily activities, relationships, work productivity, well-being, and mental health[6]. Endometriosis is usually classified according to the criteria formulated by the American Society of Reproductive Medicine (ASRM). Their criteria include four stages: minimal stage (stage I), mild (stage II), moderate (stage III) and severe (stage IV). These stages include the lesion size, location and extend of the tissue growth from the minimal to the severe stage[7, 8]. However, this staging system does not correlate between the pain symptoms and the risk of infertility[8, 9].

Endometriosis is an underdiagnosed disease that is associated with a delay from the onset of the symptoms that can take up to 7-8 years before the diagnosis[10] and the symptoms can vary widely. Women may be asymptomatic or present a single or a combination of symptoms with different intensity that can easily be attributed to other conditions[5]. This could be one of the reasons that contribute to the delay of the

diagnosis. Some of the symptoms that are associated with endometriosis include painful menstruation (dysmenorrhea), cyclical or non-cyclical abdominal pain, recurrent painful urination (dysuria), pain during and after sexual intercourse (dyspareunia), painful defecation (dyschezia), gastrointestinal discomfort and decreased libido[11]. Currently, there are no reliable biomarkers available to diagnose this disease. The gold standard for the diagnosis of endometriosis is surgical assessment by laparoscopic visualization[5].

1.2 Postulated origins

The etiology of endometriosis is complex and there are several contributing factors leading to the development of this disorder. There are numerous theories that have been put forward to explain the origin of endometriosis (Figure 1.1).

1.2.1 Sampson's Theory: Retrograde Menstruation

Retrograde menstruation is the physiological process whereby menstrual blood flows backward via the fallopian tubes into the pelvic cavity, resulting in peritoneal bleeding[12]. Sampson's retrograde menstruation theory is the most widely accepted as a cause for endometriosis. In 1927, Sampson hypothesized that endometriosis is the result of the reflux of endometrial fragments through the fallopian tubes during menstruation, with subsequent attachment and implantation of endometrial fragments, as a result forming, peritoneal and ovarian lesions[13]. Retrograde menstruation occurs in up to 90% of women within reproductive age [14] but only 6% to 10% of women develop endometriosis[1, 15].

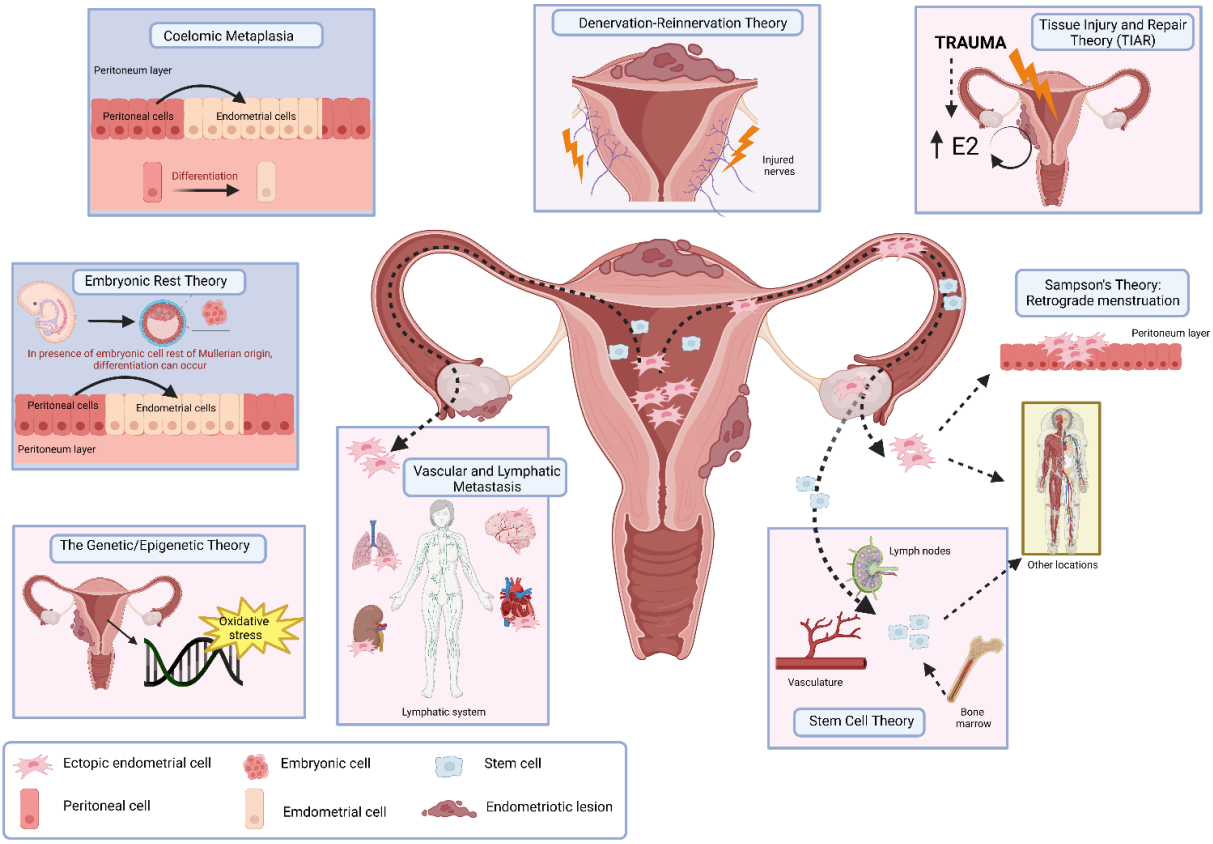

Figure 1.1: Different theories of etiology of Endometriosis. Created with BioRender.com

Although retrograde menstruation happens in most women of reproductive age, the immune system is usually able to clear the endometrial fragments avoiding its growth. However, when the immune system fails to clear the fragments, endometriosis occurs[16]. There are some hypotheses that state that endometriosis can be caused by decreased clearance of the endometrial cells and fragments due to reduced natural killer (NK) activity, and/or decreased macrophage activity[17, 18].

There are numerous factors that need further study that may play a role in the development of endometriosis, such as a dysregulation of the clearance mechanisms, altered immune system or other exogenous factors.

1.2.2 Coelomic Metaplasia

This theory was initially introduced by Iwanoff and Meyer [19, 20]. The coelomic metaplasia theory proposes the ability of normal cells, which are derivatives of the primitive peritoneum to differentiate into endometrial tissue. This theory is based on the fact that pelvic peritoneum and ovaries and Mullerian ducts are derived from epithelium of the coelomic wall[20]. This theory is used to explain endometriosis in females with the absence of menstruation, rare cases of endometriosis in males who undergo estrogen therapy for prostate cancer, adolescent girls, premenopausal women, or women with total abdominal hysterectomies[19-22]. This theory would also explain the occurrence of endometriosis in patients with Mayer-Rokitansky-Küster-Hauser (MRKH), a syndrome that consists in the lack of uterus or endometrium[23, 24].

1.2.3 Embryonic Rest Theory

This theory proposed by Von Recklinghausen and Russell in the 1890s proposes that the presence of embryonic cell rests of Mullerian origin within the peritoneal cavity could differentiate into functioning endometrial tissue under the appropriate stimuli[19, 25]. This theory also explains the presence of rare cases of endometriosis in men since the embryonic cell rests of Mullerian origin are also present in males[26] or any location along the migration pathway of the Mullerian system.

1.2.4 Vascular and Lymphatic Metastasis

In accordance with Sampson’s theory, the endometrial tissue is usually spread through the fallopian tubes explained by the concept of retrograde menstruation[13]. However, this theory does not explain implants of endometriotic tissue outside the peritoneal cavity. The lymphatic dissemination theory has been proposed to explain the

observations of endometrial tissue through the vasculature and lymph nodes [27]. Lymphatic system involvement may explain the reason why endometrial tissue can be found in rare areas outside the peritoneal cavity or ovaries such as the pleura or the pericardium[28]. In this case, understanding the role of the lymphatic system in endometriosis is essential to establish novel therapeutic approaches for the disease.

1.2.5 Tissue Injury and Repair Theory (TIAR)

The tissue injury and repair (TIAR) theory postulates that endometriosis is caused by trauma. In this context, TIAR represents an estrogen-related mechanism that is pathologically magnified in a reproductive organ that is already estrogen-sensitive[29]. The estrogen, at the same time, acts as positive feedback causing a self-perpetuation of the disease allowing the endometrial tissue to implant outside the uterine cavity[30].

1.2.6 Quinn's "Denervation-Reinnervation" Theory

The Denervation- Reinnervation theory proposes that endometriotic cells can be found outside the uterine cavity because of uterine nerve injuries and uterosacral ligaments. These may be caused by difficult intrapartum episodes and continued strain during defecation[31]. The damage of the uterine nerves causes denervation leading to a loss of fundo-cervical polarity and uterotubal dysmotility. The ectopic endometrial cells from the retrograde menstruation adhere to the damaged tissue to the peritoneal cavity and the uterosacral ligaments. The posterior tissue repair, including the reinnervation of the uterine isthmus, vaginal and uterosacral ligaments, promotes chronic pelvic pain, dysmenorrhea, and subfertility sometime after the primary injuries[32].

1.2.7 Stem Cell Theory

Stem cells are undifferentiated cells that can self-renew and generate more differentiated cells. These types of cells are usually present in the endometrial tissue[33]. Endometrial stem cells may travel to an ectopic location via different paths: retrograde menstruation from the shedding endometrium, lymphatic and vascular dissemination, direct migration and invasion or a combination of both[19].The stem cell theory postulates that the endometrial stem cells residing in the basalis layer of the endometrium[34], at menstruation, can reflux through the retrograde menstruation through the fallopian tubes and lymphatic and/or vascular dissemination establishing endometriotic lesions outside the peritoneal cavity[19, 34]. On the other hand, there are some studies that suggest that the bone marrow-derived stem cells may play a role in the development of the disease[12, 35].

1.2.8 The Genetic/Epigenetic Theory

The genetic/epigenetic theory is a more recent theory that contributes to the endometriotic disease theory (EDT)[36]. The EDT theory states that genetic events are required for the development of endometriosis. The genetic/epigenetic theory adds to this that the genetic and epigenetic changes and the redundancy of cellular processes promote changes that contribute into the diseases[37]. One of the potential causative factors to induce genetic or epigenetic changes is the oxidative stress that occurs in the uterus during the menstruation[38]. In addition to this, the redundancy of many different cellular mechanisms that can be achieved by several pathways, can explain the accumulative effect of sequential genetic and epigenetic incidents[39]. Causes of endometriosis remain largely unknown, but heritability plays a role in the genetic

component. It is estimated that heritability can contribute up to 50% with 26% due to common genetic variation[40].

1.3 Pathophysiology of Endometriosis

1.3.1 Hormonal Dysregulation

In most mammalians, endometrial functions are predominantly governed by both steroid hormones, estrogen, and progesterone. Both hormones play an important role in regulating the menstrual cycle. An imbalance in both ligands and/or their receptors can cause several gynecological disorders, including endometriosis[41]. As mentioned at the beginning, endometriosis is an estrogen dependent gynecological disorder characterized by endometrial-like tissue outside the uterus. Women with endometriosis often present high levels of estrogens while levels of progesterone are low. Although the origin of the disease is not clear, there is an obvious hormonal dysregulation associated with this disease[4].

1.3.1.1 Estrogen Dominance

The presence of estrogen plays a key role in endometriosis. Estrogen enhances the survival or persistence of the endometriotic tissue outside the uterine cavity. The four different types of natural estrogens(E2) are estradiol, estrone, estriol and estetrol. There are three major sources in the body that can produce estrogen in women with this disease (Figure 1.2): Estradiol secreted by the ovaries which can reach the attached endometrial fragments through the circulation. Aromatase in adipose tissue that catalyzes the conversion of circulating androstenedione to estrone and subsequently converted to estradiol, which can reach endometrial implants. Endometriotic tissue can also express a complete set of steroidogenic genes, including aromatase, allowing local conversion of

cholesterol to estradiol [42, 43]. Aromatase, which regulates the conversion to estradiol, produces an increase of local estrogen concentration enhancing the growth of endometriotic tissue and maintenance of endometriosis.

Estrogen binds to the estrogen receptor (ER) allowing its entrance into the cell. Estrogen acts via both ERα and ERβ. Both are expressed in human endometrial stromal and epithelial cells. Although both are present in the endometrium, ERα seems to be a more prominent mediator of the estrogenic action in this tissue, whereas ERβ is expressed at a lower level[44, 45]. Since the effects of estrogen are primarily endorsed through these receptors, their expression levels are critical in the assessment of estrogen action in endometriosis.

Some studies suggest that ERα and ERβ could act in different ways to promote the proliferation of endometrial cells and tissue-invasion activity in endometriosis sites to promote ectopic lesions. ERα might be involved during the initiation of endometriosis while the overproduction of estradiol in endometriosis would drive ERβ signaling to support the endometriotic tissue survival and inflammation[46]. Several studies have reported lower levels of ERα and higher levels of ERβ in human endometriotic tissues and primary stromal cells compared with endometrial tissue and cells[47].

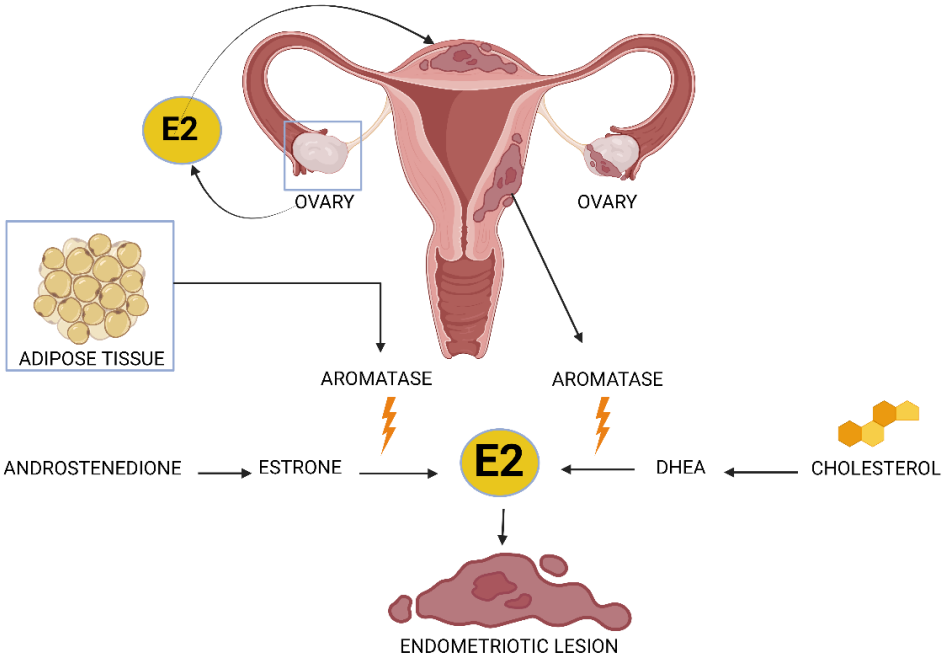

Figure 1.2: The three major sources of estradiol (E2) within endometriosis. Created with BioRender.com

Even though there are several studies seeking to understand the role of both receptors in the pathogenesis of endometriosis, there is no clear evidence of their functions during the disease. Studies using the baboon (non-human primate) as a model to study endometriosis, have shown a time dependent reduction in ERα within eutopic endometrial stromal cells a month after the endometriosis induction. This decrease was statistically significant within six months of the disease and remained throughout the time course of disease to 15 months. In the case of ERβ, this was reduced in both epithelial and stromal cells. These decreased levels of ERα and ERβ could contribute to create a non-receptive uterine environment that could affect embryo implantation[48].

1.3.1.2 Progesterone Resistance

Progesterone(P4) is a steroid hormone that is produced by the adrenal cortex and gonads, with the corpus luteum being the major source of P4 during the menstrual cycle.

P4 not only plays a key role in the female reproductive tract but plays a vital role in the maintenance of the uterus during pregnancy[49]. P4 signaling is implicated in follicular growth, ovulation and luteinization. It is also responsible for the differentiation of endometrial epithelial cells resulting in the development of secretory endometrium and the differentiation of endometrial stromal cells called decidualization to prepare the uterus for the embryo for implantation[50]. P4 signaling is compromised in women with endometriosis[51-53] . This abnormal P4 signaling in the endometrium would play a significant role affecting endometrial receptivity by interfering the regulation of the uterine epithelial proliferation and impairing decidualization [54, 55]. Another molecular consequence of the P4 resistance within endometriosis would be the increased invasiveness of endometriotic tissue promoting the establishment of the ectopic endometrial implants[55, 56].

Genetic and epigenetic studies have evidenced the notion that the eutopic endometrium of women with endometriosis respond differently to circulating P4 compared to the endometrium of women without the disease [57, 58]. This was also observed and confirmed in the baboon model of endometriosis[53, 59, 60].

The term “Progesterone resistance” is due to the failure of endometrial tissue to a properly respond to progesterone signaling [58]. Most of the actions of P4 are mediated through the binding and activation of progesterone receptors (PGR). These progesterone receptors, PGR-A and PGR-B are members of the superfamily of ligand activated transcription factors. Several studies have shown that the levels of both receptors, particularly PGR-B, are significantly lower in endometrial lesions in women with endometriosis compared with eutopic endometrium[61, 62]. In addition to this, there is

direct evidence to support that microRNA dysregulation[63, 64] and promoter hypermethylation of PGR-B[65, 66] are potential mechanisms for the loss of PGR-B in women with endometriosis.

1.3.2 Inflammatory response and immune dysregulation in endometriosis

Endometriosis is defined as an inflammatory condition. The immune system is believed to play an important role in the pathophysiology of endometriosis[67]. An altered proinflammatory immune environment in the endometrium in women with endometriosis and a lack of proper immune surveillance in the peritoneum may support the attachment and progression of endometrial tissue promoting the development of this disease[1, 50].

Endometriosis is suggested to be associated with dysfunction or suppression of different populations of immune cells. Most of them have been studied in the peritoneal cavity[68] and eutopic endometrium of women with endometriosis[69].

The next section is focused on the immune abnormalities that have been observed in patients with endometriosis. The most relevant cells, cytokines and factors involved in the progression of endometriosis are discussed below.

1.3.2.1 Proinflammatory environment: Immune cells

The immune endometrial environment is extremely dynamic. The morphology of the endometrium, proliferation, and differentiation of the cellular components during the menstrual cycle stimulates traffic of different immune cell populations[50]. Moreover, it has to be considered that not all the endometrial immune populations vary consistently during the menstrual cycle in women with or without endometriosis[69].

The leukocytes (CD45+) are distributed in the reproductive tract in either aggregated or a dispersed form in the epithelial layer, lamina propria and stroma[70].

They represent a subpopulation between 6-20% of the female reproductive tract[71]. In normal conditions, peritoneal leukocytes remove ectopic endometrial cells from the peritoneal cavity. During endometriosis, the ability to eliminate these cells is significantly decreased promoting implantation, proliferation and the recruiting and activation of peritoneal macrophages[72]. This situation facilitates an environment which may ease the progression and development of endometriotic lesions[73].

Most of the immune cells in the endometrium are cells resident in the tissue. Some of them are derived from the peripheral blood circulation. The predominant immune cells are T cells, macrophages, dendritic cells (DCs) , natural killer (NK) cells, neutrophils and mast cells[70]. These cells play an important role during endometrial remodeling and repair and facilitate the clearance of endometrial cells and tissue after menstrual shedding[74]. Some of these cells may not success in their task and allow endometrial fragments to implant at ectopic locations via retrograde flow and facilitate the establishment of endometriosis[73].

T cells

T cells are around 1-2% of the total endometrial cells[50]. T cells are one of the most important contributors to endometriosis[75]. They are part of the adaptative immune system affecting the activity of other immune cells through a variety of cytokines secreted by these cells [50, 76].

Different subtypes of T cells are involved in the immune response of endometriosis through different mechanisms[75]. Depending on the surface marker and function, different types of T cells have been identified. The most relevant in endometriosis are T helper cells (Th) and regulatory T cells (Treg). Both express CD4 and each of them are

characterized by the type of cytokine that they produce. Some of the subcategories that are included in the T helpers are Th1, Th2 and Th17[77].

Th1 cells stimulate the production of IL-2, IFN-γ and lymphotoxin, and other cytokines that promote the action of macrophages and natural killer[78] cells. There is a lack of knowledge regarding Th1 in the endometrium of women with endometriosis. In peripheral blood, Th1 cells are more abundant in women with endometriosis when compared with women without the disease[79]. The presence of these cells is associated with the severity of the disease, more specifically, with the secretion of IL-2 and IFN-γ that have been found to be considerably elevated in women with deep infiltrating endometriosis[78].

Th2 stimulates the production of IL-4, IL-5, IL-6, IL-10 and IL-13. IL-4 and IL-10 which are cytokines that are known to collaborate with B cells, triggering humoral immune responses which recruit and activate other cells to sites of inflammation[78] . IL-4 and IL-13 are also cytokines involved in the differentiation of resident fibroblasts into myofibroblasts. This suggests that Th2 cells may be involved in the process of fibrosis[80], which is one of the hallmarks of endometriosis.

Th17 are cells derived from naïve CD4+ lymphocytes in the presence of TGF-β and IL-6[80]. These T helpers are characterized by the production of cytokines such as IL-17, IL-17A, IL-21, IL-22[81]. They are present in blood and the peritoneal fluid at different stages of endometriosis. The percentage of Th17 cells in the peritoneal fluid in women with advanced stages of endometriosis has been shown to be higher than in women with mild endometriosis[82]. They appear to contribute to disease severity and promote the proinflammatory environmental characteristic of endometriosis[50]. Some

studies have shown that the elevated levels of IL-17A, in the plasma and peritoneal fluid of women with endometriosis could play an important role in the progression of endometriosis stimulating the production of other cytokines that are involved in the process of the angiogenesis and chronic inflammation[83, 84].

Regulatory T cells (Treg) are an important population of cells that promote the proinflammatory environment in women with endometriosis. They can regulate the Th1/Th2 response[85], macrophages, mast cells and natural killer cells amongst other types of immune cells[69]. They are present in ectopic lesions and in the peritoneal fluid of women with endometriosis, appear to be increased in with women who suffer from endometriosis compared with women without the disease. This has also been observed in the baboon mode[86]l. Some studies have shown that Treg cells in the peritoneal fluid have a positive correlation with the stage of the disease[87].

Macrophages

Macrophages play an important role in the immune response. They are capable of stimulating the synthesis and secretion of a variety of molecules that can alter the function of neighboring cells[73]. Endometriosis is an inflammatory disease, in part, due to the abnormal function of the macrophages. Activated macrophages can secrete IL-1, IL-6, IL-8, IL-10, IL-12 and IL-13 amongst others[50]. The two main phenotypes to which macrophages can polarize are the classical phenotype (M1) and the alternate phenotype (M2)[88]. M1 are known to secrete cytokines that promote the initial response against infections and triggering the inflammatory response. On the other hand, M2 present as an anti-inflammatory and tissue, primarily promoted by IL-4, IL-10, IL-13 and transforming growth factor-β (TGF-β). Studies have shown that M2 macrophages are involved in tissue

repair during inflammation mainly in the peritoneal environment of women with endometriosis[89-91].

When considering that the macrophage phenotypes in women with endometriosis compared to women without endometriosis are different. Also, the complexity of the nature of the endometriotic lesion environment, it is most likely that the macrophage M1-M2 polarization in endometriosis may depend on a variety of functional states in the disease[88].

Dendritic cells (DCs)

Dendritic cells are antigen presenting cells essential for the initiation and maintenance of the T cells immune response[73]. They produce cytokines such as IL-6, IL-10, RANTES among others that have an important role in the proinflammatory environment[92] especially in endometriosis. There are studies showing that activated DCs are significantly higher in women with endometriosis with stage I-II, compared with women without the disease that could indicate that these cells are critical players in the development of immune response[93]. DCs can play a role in endometriotic lesions increasing the neuroangiogenesis and contributing to the lesion growth and pain in women with this disease. In general, immature DCs are higher in eutopic endometrium in women without endometriosis, suggesting a potential failure in DC maturation in women with endometriosis which could lead to a failure in the clearance of the endometrial cells shed during menstruation, promoting progression of the disease[50].

Uterine Natural Killer (uNK) cells

uNK are the predominant leukocyte population in the normal endometrium. They play an important role, not only in endometriosis, but also in infertility[94]. Different studies

have shown that changes of uNK cells in the blood, peritoneum and endometrium, could play a role in the development of endometriosis[95]. These cells, in normal endometrium, have low cytotoxic activity. However, in women with endometriosis their activity is even more reduced than in the normal endometrium. In cases where women with endometriosis also suffer from infertility, or present with recurrent pregnancy loss, the uNK cytotoxic activity is even higher. This suggests that uNK play an important role during the maintenance and the establishment of pregnancy[50].

1.3.2.2 Cytokines and growth factors

The cascade of events that involve the inflammatory response is an important aspect in the development of endometriosis. As previously mentioned, there is clear evidence that endometriosis is related to an abnormal function of different immune cells that produce specific cytokines. Cytokines and growth factors are proteins produced by immune cells that are usually increased in the peritoneal fluid of women with endometriosis, that contributes to the pathogenesis of the disease[96]. Some of the cytokines that seem to play a crucial role in the establishment and lesion survival in endometriosis are described below.

Interleukin-6 (IL-6)

IL-6 is one of the most studied proinflammatory cytokine in endometriosis and is one that is highly expressed in patients with endometriosis[97-99]. It is primarily produced during acute and chronic inflammation. IL-6 is increased in the endometrium of women with endometriosis and in ectopic lesions[69, 100]. It affects the secretion of other cytokines, promotes the activation of T-lymphocytes and the proliferation of B-lymphocytes[101]. Macrophages can secrete IL-6 in response to different substances in

the peritoneal fluid. Endometrial epithelial and stromal cells produce IL-6 depending on the hormonal and immunological environment[102]. Increased levels of IL-6 in the peripheral blood of patients with endometriosis also suggests that this could be an important cytokine that could be used as a serum marker for non-surgical prediction of endometriosis[103].

Interleukin-8 (IL-8)

IL-8 is an activator of neutrophils and a very potent angiogenic cytokine[104]. There are several studies that have shown increased concentration of IL-8 in the peritoneal fluid and the serum of women with endometriosis when compared to women without the disease. It stimulates adhesion of endometrial cells to fibronectin, implying a role for it in the pathophysiology of the disease by promoting cell attachment and proliferation. It has also been observed that the elevated IL-8 levels in women with endometriosis might be correlated with the severity of the disease[105].

Interleukin-1 (IL-1)

IL-1 is mainly produced by monocytes and macrophages and plays an important part during the regulation of the inflammation and immune response. The IL-1 family consists of two different molecular forms, IL-1α and IL-1β[106]. IL-1 is a pleiotropic cytokine that may promote the development of endometriosis in the peritoneal cavity upregulating the expression of other cytokines such as IL-6 or other growth factors. Also, it has been shown that the IL-1α is a potent stimulator of matrix metalloproteinase enzymes (MMPs) during the proliferative phase in the endometrium[107]. IL-1 increases the secretion of MMP-1, MMP-2, MMP-3, MMP-9 in uterine endometrial cells from women with endometriosis[106].

Tumor necrosis factor alpha (TNF-α)

TNF-α is a pleiotropic cytokine that plays an important role during the inflammatory process involved in the progression of endometriosis. It is mainly produced by macrophages, uterine natural killers, Th1 cells together with other cells[69]. In the human endometrium, TNF-α is implicated in the physiological process of endometrial proliferation and shedding[108]. It has also been observed that it can activate inflammatory leukocytes and stimulate macrophages to produce other cytokines such as IL-1 and IL-6, which further enhances the proinflammatory environment in women with endometriosis. In addition to this, it induces the expression of other enzymes, such as COX2, which can stimulate cell proliferation and angiogenesis, promoting endometrial tissue growth[69]. Studies have reported the presence of significantly higher levels of TNF-α in patients with endometriosis at an early stage of the disease[109]. TNF-α may be one of the essential players for the pathogenesis of endometriosis development.

Transforming growth factor-β (TGF-β)

TGF-β is an inflammatory growth factor that plays an important role during the development of endometriosis. Different studies have shown an increase of this growth factor in the peritoneal cavity of patients with endometriosis when compared to women without the disease, implying a role during the pathogenesis of the disease[110, 111]. TGF-β is one of the most widely studied molecules in fibrosis. There are three different isoforms (TGF-β1, TGF-β2 and TGF-β3). Even though there are a variety of cell types that respond and produce TGF-β, TGF-β1 is the one that has been mainly associated with the development of fibrosis[80]. TGF-β is a very powerful chemoattractant growth factor for macrophages, leukocytes and fibroblasts[101]. Some studies have reported that

increasing levels of *TGF-β* in the peritoneal cavity of women with endometriosis could be associated with the increased survival, invasion and proliferation of ectopic endometrial cells during endometriotic lesion development[112].

1.3.3 The fibrotic component during endometriosis

During retrograde menstruation, the process of the menstrual tissue travelling into the pelvic cavity can result in peritoneal and ovarian disease followed by inflammatory responses, scarring and fibrosis[113]. Fibrosis is defined by the development of fibrous connective tissue resulting from continuous tissue damage and repair.

Myofibroblasts are crucial to the process of fibrosis. Once these cells are activated, they proliferate and produce a collagenous extracellular matrix that heals but at the same time, disrupts and modifies the surrounding structures. This process is defined surgically as scarring[114]. The activation of myofibroblasts in endometriotic lesions can be detected based on the presence of α-smooth muscle actin (α-SMA). During the development of fibrosis, the lesions can lose characteristic features of endometrial glands and stroma complicating the histologic confirmation of the disease.

There are two factors that seem to be critical for the activation of myofibroblasts: Transforming growth factor and the stiffness of the tissue[115]. When the myofibroblasts are activated, they can induce an increase in proliferation, migratory ability, production of cytokines and interstitial matrix that stimulates changes in the environment. Continuous myofibroblast activity causes accumulation and contraction of collagenous extracellular matrix enhancing fibrosis. This process eventually will result in a hypertrophic scar, causing the disruption of the anatomy of the tissue[116].

1.3.3.1 Fibrosis in endometriotic lesions

The peritoneal cavity is one of the most common areas where endometrial lesions can be established. They contain an abundant amount of smooth muscle that seems to represent an important characteristic of peritoneal endometriotic lesions. As it was previously mentioned, TGF-β levels are significantly increased in the peritoneal fluid of women with endometriosis when compared to women without the disease. One of the events that has been observed in mesothelial cells is that when they are exposed to TGF-β, the production of lactate is increased which produces a reduction of the pH of the environment. This increase in the production of lactate promotes the acidic activation of the TGF-β ligand causing a secondary induction of myofibroblast differentiation[117].

A different type of lesions are the ovarian cysts. It is known that fibrosis can be present in the ovarian cyst walls. The pseudo-capsule of the cyst is primarily constituted by fibrotic tissue. Fibrosis can also be identified in the ovarian cortex surrounding the endometrioma. Some studies have also shown that cells derived from ovarian endometriosis activated platelets can promote epithelial to mesenchymal transition (EMT), fibroblast to myofibroblast transdifferentiation and differentiation to smooth muscle cells. This resulted in an increase in the cell contractibility, collagen production and fibrosis, via TGF-β/Smad signaling[118].

1.3.4 Infertility

Infertility is defined as the inability of a couple to conceive after one year of unprotected intercourse. Infertility is one of the primary concerns related to endometriosis. In healthy couples, the probability of achieving pregnancy in any single month is around 15-20%. In women with undiagnosed or untreated endometriosis, this number is closer

to 2-10%[101]. It is estimated that 35-50% women with this disease are infertile[1] and that 25-30% of all infertile women have endometriotic lesions as the only recognizable cause of their infertility[119]. Women with endometriosis have a higher risk of infertility and miscarriage due in part to endometrial abnormalities[120]. The distorted anatomy that scarring and the local inflammation can cause, could be some of the reason that can lead to infertility[50].

The distortion due to the scarring and fibrosis in combination with the inflammatory response, promote a hostile environment in the pelvic cavity and uterus that contributes to infertility in patients with endometriosis by affecting oocytes, sperm, and embryos. In addition to this, women with this disease who achieve pregnancy have worse pregnancy outcomes than women without endometriosis. This could be a consequence of the proinflammatory environment previously described in the endometrium and the effects during the processes of nidation and placentation[121]. Other possible causes of infertility in women who suffer endometriosis could be: adhesions, disturbed folliculogenesis, luteal phase defects, progesterone resistance and anti-endometrial antibodies[122-124]. Despite a clear association between endometriosis and infertility, some of the mechanisms connecting them remain unclear.

In the literature, there are several tools, including the endometriosis fertility index (EFI), that help predict pregnancy rates in patients with surgically documented endometriosis who attempt non-IVF conception. This tool is helpful only for those infertility patients who have had surgical staging of their disease, but it is not designed to predict any aspect of endometriosis-associated pain. EFI provides a score from zero to ten and the score predicts results from subsequent non-IVF treatments[125].

There are several studies that support the association between endometriosis and infertility even in early stages of the disease. Nearly 50% of women with minimal or mild endometriosis, will be able to achieve pregnancy without any treatment. However, only 25% of women with moderate endometriosis, will conceive spontaneously, while only a few spontaneous conceptions will occur in severe cases of endometriosis[126]. Superficial peritoneal lesions are more closely related to infertility than cases of deep infiltrating endometriosis[122]. Studies with animal models have also been able to replicate the association between endometriosis and subfertility. Studies that include the rabbit as an animal model, have been able to show that injecting peritoneal fluid from animals affected by endometriosis into normal animals, results in a decrease in their implantation sites implying that substances in the peritoneal cavity of the animals, affected by endometriosis, could impact fertility [127]. The same effect was observed in fertile mice when they were injected with human peritoneal fluid from infertile women with endometriosis[128]. In addition to this, studies using the mouse model suggest that the development of endometriosis can cause implantation failure and a defect of decidualization, as occurs in humans, impacting fertility[129]. Furthermore, data from the baboon model suggest that in the induced model of endometriosis in the baboon, an increased angiogenic capacity, decreased apoptotic potential, progesterone resistance, estrogen hyper-responsiveness, and an inability to respond appropriately to embryonic signals contribute to the reduced fecundity associated with this disease[130].

As previously mentioned, peritoneal factors play an important role during the development of endometriosis. The inflammatory environment associated with macrophages and the production of cytokines can affect processes such as ovulation,

embryo quality and implantation[101]. As described before, IL-6 plays an important role in endometriosis. It is a cytokine that is normally low during the proliferative phase and high during the implantation window. Some studies have shown that the expression of IL-6 was reduced in a group of women experiencing unexplained recurrent miscarriages when compared to fertile women[131].

Another aspect of endometriosis to consider is the P4 resistance that occurs during the development of the disease. P4 and E2 are essential for the establishment of a successful pregnancy. The progesterone resistance and dysregulation of hormonal signaling during the development of endometriosis, could be another contributing factor that impacts during implantation by altering the endometrium and affecting fertility[55]. Embryo implantation is a rigorously controlled process that is regulated by E2 and P4 in the majority of mammals[132]. A balance between E2 and P4 is required to achieve a successful implantation. The progesterone resistance that occurs in endometriosis could interfere with the expression of progesterone-induced proteins critical for implantation and endometrial receptivity that could contribute to infertility related to endometriosis[133].

TFG-β, is growth factor that is crucial for female reproduction. A dysregulation of the TFG-β signaling pathway may have catastrophic consequences, leading to reproductive diseases, disturbing embryo implantation, ovulation or decidualization[134]. There is evidence that TFG-β may play a role in the etiology of endometriosis[135]. TFG-β is also known as a mediator of P4 action during the secretory phase[136] and modulates expression of critical proteins, including leukemia inhibitor factor (LIF), implying a mechanism by which fertility might be compromised[101].

It is known that fertility is compromised in some women with endometriosis, even at the early stages, while other women have minimal effects. As previously mentioned, the balance between E2 and P4 is crucial for a normal implantation and the loss of this equilibrium during endometriosis could be one of the causes for infertility. Management of infertility caused by endometriosis will be discussed later in this chapter.

1.4 Role of microRNAs during Endometriosis

Different studies have suggested that microRNAs(miRNAs) play a role in non-malignant and malignant diseases that involve the human reproductive tract. Abnormal expression of different miRNAs has been observed in several reproductive tract diseases including preeclampsia, endometrioid endometrial adenocarcinoma and endometriosis[137, 138].

1.4.1 Biogenesis of miRNAs

miRNAs are small, noncoding regulatory RNAs that are not translated into proteins[139]. miRNAs contain ~20 nucleotides that can regulate gene expression and play a fundamental regulatory role in several pathological processes[140-142]. In 1993, Rosalind Lee, Rhonda Feinbaum and Victor Ambros presented the first evidence of what is now known as miRNAs. Ambros's group described a 22 nucleotide RNA encoded by lin-4, a gene in *Caenorhabditis elegans* involved in larval development that does not code for a protein, but instead can bind to the lin-14 transcript and regulate its expression[140, 142, 143]. In the canonical miRNA biogenesis pathway (Figure 1.3), genes are transcribed by RNA polymerase II or RNA polymerase III to produce a primary microRNA transcript (pri-miRNA). This transcript, still in the nucleus, is processed into a smaller precursor miRNA (pre-miRNA), by the microprocessor complex Drosha-DGR8. The

resulting precursor is translocated from the nucleus to the cytoplasm by Exportin-5-RAN-GTP. Once there, Dicer-TRBP complex cleaves the pre-miRNA into a mature single-stranded miRNA. The mature miRNA binds to its mRNA target at their complementary sequence to reduce the expression of the target protein by inhibiting mRNA translation to proteins or simply by decreasing the mRNA levels[139, 144, 145].

The study of miRNAs is crucial for the understanding of the pathophysiology of different diseases. Over the years, several studies have reported that the altered expression of miRNAs is associated with cancer, cardiovascular diseases, diabetes, fibrosis and gynecological diseases such as endometriosis[146].

1.4.2 Relevant miRNAs in endometriosis

Studies of miRNA expression support the hypothesis that miRNAs play an important role during the progression of endometriosis[137]. Some miRNAs such as miR-29c, -451, -141, -21, -200a have been shown to be altered in baboons with endometriosis. These studies results were also validated in women with endometriosis[147]. miRNAs can be involved in processes such as progesterone resistance, inflammation, cell proliferation, extracellular matrix remodeling and angiogenesis among other processes that are key for the development of endometriosis[101].

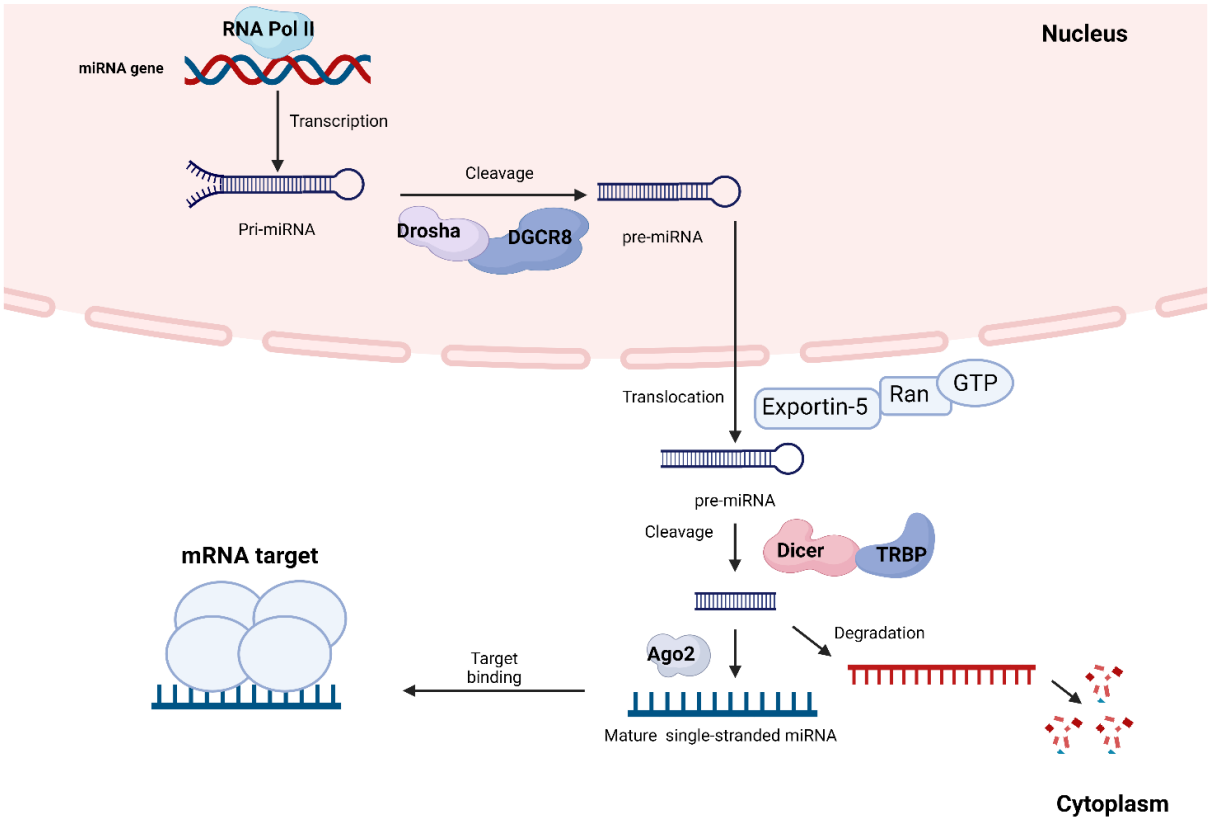

Figure 1.3: Canonical miRNA biogenesis pathway. Created with BioRender.com

Some miRNAs have been shown to be associated with infertility. MiR-29c has been demonstrated to contribute to progesterone resistance. This miRNA is upregulated in endometriosis, decreasing the expression of one of its targets, the FK506-binding protein 4 (FKBP4) gene. FKBP4 is a co-chaperone that optimizes the function of the progesterone receptor. The upregulation of miR-29c resulting in the decrease of FKBP4 during the window of implantation, could lead to progesterone resistance in women with endometriosis, promoting infertility[53].

Another miRNA that has been reported to be altered in endometriosis in baboon studies and in women with endometriosis is miR-451. This miRNA, through its targets, is involved in the suppression of apoptosis and cell proliferation and invasion, which are hallmarks of endometriosis[147]. The downregulation within endometriotic lesions of this

miRNA leads to significant increases in the expression of its predicted target, YWHAZ. YWHAZ codes for the protein 14-3-3ζ, known to suppress apoptosis, enhance proliferation and promote invasion which are hallmarks of endometriosis[147].

In endometriosis, miRNAs that are expressed have control over oncogenic proteins that are involved in cell growth. MiR-143 is highly expressed during endometriosis, inhibiting cell proliferation[101].

Another major process that has been studied related to miRNAs and endometriosis is angiogenesis. MiR-15a-5p was reported to be suppressed in endometriotic lesions and proposed to be involved in angiogenic events associated with endometriosis[148].

1.5 Management and treatment in current clinical practice

Various studies have described different medical and surgical therapies for endometriosis. Laparoscopy is currently the gold standard to visualize lesions and detect endometriosis, but ideally, it should be linked with a confirmed positive histology[149]. Treatment for endometriosis includes two important points: pain relief and amelioration of infertility. In some cases, medical therapies that are used to manage pain, in general, are not useful to manage infertility[150].

When the goal is to enhance fertility, medical treatment is not the best option. They are hormonal and mainly focus on blocking ovulation and estrogen production. When the main goal is managing pain, medical treatment is one of the best options, although surgery might also be a possibility depending on the severity of the pain in the patient[5].

1.5.1 Management for pain

Endometriotic lesions are often associated with underlying fibrosis and distortion of the surrounding anatomy that can lead to pain[151]. The sensitivity to E2 in

endometriosis involves the growth of nerve endings into the endometrial lesions which could also have an influence on the activity of the neurons throughout the central nervous system leading to pain[152]. Depending on the severity of the pain, medical and/or surgical procedures might need to be implemented.

As endometriosis is an estrogen dependent disease, medical treatments have been focused on establishing a hypo-estrogenic or hyper-progestogenic environment[5]. It is important to mention that medical treatment usually does not eradicate the disease and chronic pelvic pain associated with endometriosis involves repeated courses of medical therapy, surgical intervention, or both. In most cases, the symptoms reappear after the therapy is discontinued[11].

During management of the pain, the desire of the endometriosis patients to conceive naturally is not usually immediate. In this case, combined oral contraceptives pills containing estrogen and progestin are provided to the patients. These contraceptives induce a central inhibition of gonadotropin secretion, inhibiting ovulation and reducing ovarian estrogen secretion. In this scenario, the combined oral contraceptives promote a hyper-progestogenic environment, inducing decidualization and atrophy of the ectopic endometrium[153]. Gonadotropin-releasing hormone (GnRH) agonist can also be administered to suppress hypothalamic and pituitary function producing a hypo-estrogenic state. They are usually very effective to treat pain[154]. The most widely used agents are GnRH agonist or antagonists and oral contraceptives[1].

The surgical approach to relieve pain in endometriosis can sometimes be used as a first option or initiated following failed medical therapy[2]. Surgical procedures usually include excision, laser ablation of endometriotic implants on the peritoneal surface,

excision or drainage or ablation of endometriomas and interruption of nerve pathways[2]. This conservative surgery (no removal of the ovaries or uterus), is usually performed in women who wish to keep their ability to conceive. The aim of this approach is to eliminate all visible endometriotic lesions and try to restore the anatomy. Although the effect on the pain after performing this type of surgery is usually satisfactory, symptoms may reoccur after surgery[155]. However, it does not always imply the recurrence of the disease.

For those endometriosis patients that suffer from pain and the desire to conceive is no longer an issue, hysterectomy or a bilateral salpingo-oophorectomy is also an option. Hysterectomy for chronic non-specific pelvic pain associated with endometriosis is an alternative for many women[156]. Some studies have observed a significant long-lasting reduction in pain symptoms after this procedure[157].

1.5.2 Management for infertility

The treatment options for women with endometriosis related to infertility are usually medical treatment, surgery or assisted reproductive techniques. The use of hormonal treatments use for pain management are usually contraindicated due to their contraceptive effects[122]. There are two main medical strategies to approach infertility. One of them is the suppression of follicle growth with the goal of inducing amenorrhea and suppressing development and growth of endometriotic lesions, increasing fertility. The other option is through the stimulation of follicle growth and ovulation[122].

Surgery is another option for infertility management associated with endometriosis. Ablation of endometriotic lesions is recommended for treatment of infertility related to stage I or II endometriosis[149]. In moderate or severe endometriosis, the goal of the surgery is to restore normal anatomy of the pelvis and remove large endometriomas[122].

The benefit of medical treatment before or after surgery is not clear, but the suppression of endometriosis before surgery may promote the reduction of inflammation. In women with ovarian endometriomas undergoing surgery for infertility or pain, depending on the methodology used for its management, can increase the spontaneous post-operative pregnancy rate[158].

In any case, the risk and the benefits associated with surgical procedures for endometriosis associated with infertility must be evaluated carefully.

Assisted reproductive techniques (ART) such as IVF can bypass the fallopian tubes increasing the chances of pregnancy. It is currently the most successful treatment for those cases of infertility associated with endometriosis. These techniques are highly recommended, especially in cases where the tubal function is compromised[159].

1.6 Animal models to study the disease

In vitro models have been a great alternative to study endometriosis, but due to the considerable limitations, animal models are indispensable to study the mechanisms of this disease. For years, animal models have allowed us to investigate mechanisms and regulation of pathways of different diseases in a controlled manner. While there are known disadvantages of extrapolating data across animals and humans, animal models have been useful to study different events involved in the etiology and pathophysiology of endometriosis[160].

Non-primate animal models of induced endometriosis have been crucial in the study of endometriosis. Some of the animals in this group include rabbits[161-163], rats[164-166] and mice[167, 168]. These models are used to study mechanisms involved in endometriosis that are not viable to perform in larger mammals. Also, the animal care

facilities, handler training, and housing are more cost effective and manageable than larger animal models.

Several non-human primate models, including macaques[169, 170], and baboons[171], have been extremely useful to study endometriosis. They have the advantage of providing a phylogenetically similar animal model to the human. In the case of the baboon model, this animal has an endometrial morphology, physiology, and menstrual cycle similar to women[18]. Baboons can also develop spontaneous endometriosis which makes them one of the most suitable and appropriate models to study this disease[171, 172].

The next section is primarily focused on baboon and mouse models for endometriosis.

1.6.1 Baboon model

The development of animal models of endometriosis has been, and still is, crucial for the investigation of disease pathogenesis and development of new targets for treatments[173]. The mechanisms underlying the development and the maintenance of this disease remain unclear. This lack of understanding, as well as the human ethical considerations and limitations, increases the demand for a suitable model to study this disease[101].

Although none of the current animal models have been completely successful in mimicking all the aspects of this disease, they have been proven to be very valuable tools. They have been key models to design controlled studies with the goal of better understanding this disease and to develop better methods for an early diagnosis of endometriosis[172].

The non-human primate model of endometriosis, particularly the baboon, has demonstrated an unquestionable relationship between women with this disease, revealing similar reproductive physiology, immunology and establishment and progression of endometriosis[171, 172]. Although the cost of maintenance for these animals is more expensive than most other species, the baboon offers clear advantages for crucial studies focused on endometriosis[171].

Along with this, baboons can spontaneously develop endometriosis resembling the ectopic lesions in humans[174]. These lesions have been found in the baboon to range from minimal to disseminated form, like the different stages of disease in women[171, 175]. Other advantages of the use of the baboon as a model is that endometriosis can be induced by injecting autologous menstrual tissue into the pelvic cavity of the same animal. The injection of the menstrual tissue into the intraperitoneal cavity mimics the physiological process of retrograde menstruation which also allows the study of the progression of the disease from its very initial stages[48, 171, 174]. In addition to this, the baboon permits multiple and complex surgical procedures and collection of biological samples during the time frame of the disease without the need for a hysterectomy.

All these advantages, make the baboon an excellent model to study the pathogenesis of endometriosis (Supplementary Table 1A), including the identification of components that may play a role in the establishment of the disease and the development of endometriotic lesions. All these advantages can provide a clearer understanding of the mechanisms that contribute to the alteration of the endometriotic environment that could result in pain, fibrosis, or lead to infertility in the context of endometriosis.

This primate also offers an important preclinical model to test drugs for the prevention or treatment of the disease. Studies to evaluate therapeutical effects can alternatively be performed in animals that already have the disease (spontaneous or induced) allowing the performance of both paired (before and after treatment) and unpaired (treatment vs. positive control and negative control) comparisons[172].

Taking into consideration all these advantages, the baboon model has been utilized in a significant number of studies[48, 99, 147, 176-190]. Some of these studies include the assessment of the efficacy of statins, such as simvastatin, in the baboon model with endometriosis. The statins represent an important alternative approach for the potential treatment of endometriosis for women for whom hormonal treatments are ineffective or cause undesirable effects[191].

In addition to this, other studies have also used the baboon model of endometriosis to validate aberrant miRNAs expression in the pathogenesis of endometriosis[53, 147]. This has not only been helpful to validate the altered expression of miRNAs in baboon and human tissue, it has been also crucial for those studies that have demonstrated differentially expressed miRNAs levels in serum in patients with endometriosis and have been focused on circulating miRNAs as regulatory markers in serum for this disease[192, 193].

1.6.2 Mouse model

As mentioned previously, humans and certain primates, such as baboons, have similar reproductive biology. They have similar menstrual cycle length as well as cyclical endometrial changes, and embryological similarities[194]. Although there are strong

advantages to using the baboon model of endometriosis, the cost can be extremely expensive and limit the options for several researchers.

Due to this, the mouse model of endometriosis has also been critical for the study of the disease. They are a more economical model and logistically more attractive than the baboon model[168]. The mouse model provides an avenue to investigate multiple aspects of the disease at multiple time points. In addition to this, the rodent model does not impose the same ethical restrictions as in humans or non-human primates[101]. Although they are non-menstruating species, and do not develop endometriosis spontaneously, compared to humans or the baboon model, rodent models have been and still are extremely useful for transgenerational studies to understand the potential impact of endometriosis in women with this disease.

There are immune responses and hormonal regulation that play an important role in the pathogenesis of endometriosis that differ in mice and humans that could present some challenges; however, the mouse model is widely used due to the numerous advantages. One of them is the small size, the short estrous cycle and the length of their gestational time allowing them to have new offspring once per month. In addition to this, the ease of genetic manipulation and regulation of target specific genes make the mouse a well suited model for dissecting and investigating different pathways of disease pathogenesis[167].

After the surgical induction of endometriosis in the rat which Vernon and Wilson described in 1985 [195], Cummings and Metcalf reported in 1995 one of the first mouse models of endometriosis using a similar method [196]. Since then, there have been

multiple studies that describe the generation of mouse models that can replicate the molecular hallmarks and features of endometriosis[167].

1.6.2.1 Induction methods for the development of endometriosis in rodents

As mentioned previously, mice do not menstruate. To facilitate the study of endometriosis, the ideal mouse model would be the one that presents a mechanism that allows the endometrial tissue to translocate to the peritoneum via retrograde menstruation as initially proposed by Sampson in 1927[13]. Currently, there is no such model, but there are others that have been developed over the last several years that facilitate the study of endometriosis in the mouse model. Different methods of induction of endometriosis have also been developed.

Suture method: The suture method consists of the suturing or adhesion of uterine tissue into the peritoneal cavity of a recipient or the same mouse. This endometriosis model allows for the easy location of the lesions and measurement of their sizes. This model also allows the use of autologous, syngenetic, or heterologous engraftments.

Autologous and syngeneic models use murine tissue donors. The uterine tissue engrafted is usually sutured to the peritoneal wall[196, 197]. It is generally performed with nylon, silk or braided silk sutured to the peritoneum and the sizes of the pieces can vary from 2 to 5 mm. The placement of the pieces is usually in the peritoneum, but other locations such as intestines, bowel mesentery or ovaries, could also be designated to place the engraftments[198-201]. The type of lesions collected using this method display lesions like what is observed in humans. These lesions tend to form cysts, being filled with liquid and present with histological evidence of glands and stroma[202-204]. This

model has been extensively used to investigate different aspects of the pathogenesis of endometriosis such as angiogenesis or growth of ectopic lesions.

The suture method has advantages but also has several limitations. One of the challenges that presents is that surgically implanted lesions completely bypass the attachment phase of the disease. This model does not allow for the study of spontaneous lesion attachment or varying the location of development of the lesion[167]. In addition, the suture material and the healing process associated with this type of surgery may alter the normal process of lesion development and may alter how angiogenesis develops[205].

Injection method: The injection method involves an injection of uterine fragments through a surgical opening into the peritoneal cavity. This method consists of the introduction of minced tissue taken from one of the uterine horns of the same mouse or a donor mouse. The uterine horn is cleaned by stripping all the fat and muscle. The pieces are usually less than 1.5 mm[206, 207].

Normally, the minced uterine fragments are suspended in a solution that mimics the pH of the peritoneal cavity to keep the viability of the uterine fragments before the injection into the peritoneal cavity. Usually, the suspension volume is between 200-500ul of PBS but the solution or the volume can vary depending on the study. After this procedure, the wound is closed with a suture and followed by wound clips. Massaging the area where the fragments have been introduced usually helps to spread the tissue around the peritoneal cavity[207].

Following surgery, the recipient mouse can develop multiple lesion types. Typically, lesions will form around the injection site, and they may even form a cesarean

scar. Lesions are typically light pink or cystic, but white fibrotic lesions can also be found. The lesions are usually found attached to the peritoneal wall, intestinal mesentery, behind the stomach or spleen and perivesical adipose tissue[102].

One of the advantages of this method is that it allows the study of the initial stages of endometriosis, which includes the process of angiogenesis, defective apoptosis, endometrial proliferation, and the inflammatory environment[208].

1.7 Outlook for the research on endometriosis

Despite years of research, our knowledge of endometriosis and the ability to successfully treat it, is delayed by the incomplete understanding of the pathogenesis of the disease[209]. The diverse clinical and molecular aspects of endometriosis likely due to inconsistencies in the disease classification and the variability among control populations remain uncertain.

New approaches in the field of endometriosis such as RNA-sequencing (RNA-seq)[210], transcriptome analysis[211], or single-cell RNA-sequencing (scRNA-seq)[212, 213] could help to characterize this heterogeneity among the population and facilitate the assessment of gene expression during the disease compared without it. RNA-seq has been increasingly considered as a very popular technique within the field of endometriosis. RNA-Seq studies have become the gold standard for high-throughput transcriptome analysis. The amount of data produced provides the opportunity to analyze gene expression or even aspects related to biological roles of small RNAs including miRNAs[214]. Different studies have used the “-omics” technologies to investigate the gene expression profile of the genome of the eutopic endometrium from women with endometriosis[93, 215, 216].

In this dissertation, we also acknowledge the importance of the new approaches that provide RNA-sequencing and transcriptome analysis in the field of endometriosis. We used the mouse model to explain the component that intervenes in the early lesion development. The studies performed will contribute to the current field of endometriosis by understanding some of the mechanisms of inflammation and fibrosis during the development of this disease. We hypothesized that the inflammatory environment driven by IL-6 can upregulate miR-21, promoting the development of fibrosis during the progression of endometriosis.

After this introductory chapter 1, chapter 2 focuses on the characterization of the transcriptome of endometriotic lesions using the mouse model. The outcome of this chapter highlights the important role of genes involved in inflammatory processes and development of fibrosis. Chapter 3 provides evidence that miR-21 participates in the development of fibrosis during the progression of endometriosis. Chapter 4 elucidates the mechanisms by which miR-21 is regulated by IL-6 inflammation with one of the causes of fibrosis. Chapter 5, the final section of my dissertation, summarizes and discusses the overall studies and proposes future directions based on other interesting findings from the transcriptomic analysis.

CHAPTER 2: CHARACTERIZATION OF THE TRANSCRIPTOME OF ENDOMETRIOTIC LESIONS

2.1 Introduction

Endometriosis is a chronic inflammatory disease defined as the presence of endometrial tissue outside the uterine cavity[217]. It is estimated to affect roughly 10% of reproductive age women globally, which is approximately 190 million women worldwide[218]. Despite affecting this number of women globally, the pathophysiology and economic impact of the disease are not completely understood[219].

The overall direct medical cost to a patient with endometriosis per year in 2022 was from US$1,459 to US$20,239, with surgery being the main contributing factor. The indirect costs of endometriosis have oscillated between US$4572 and US$14,079 in the past years. These indirect costs include work loss, disability and reduced productivity[219]. Despite the significant effect that endometriosis has on women, and the economic burden for the healthcare system, the etiology of this disease still remains unclear.

The most widely accepted theory about the origin of endometriotic lesions is that it is likely derived from retrograde menstrual tissue. This term refers to the reflux of menstrual debris containing viable endometrial cells travelling through the oviducts and reaching the peritoneal cavity, adhering and invading the underlying mesothelium[220]. Although most of women in reproductive age experience retrograde menstruation, only a few develop endometriosis[1]. This could suggest that the eutopic endometrium in women with endometriosis could alter the characteristics of the endometrial cells or the properties

of endometrial cells that are shed into the pelvic cavity, including their tendency to implant and proliferate, and play an important role in the establishment of the disease[221].

In addition to these possibilities, a malfunction of the immune system may also interfere with the clearing process of the implanted tissue[2]. Several studies have shown that this process can elicit an inflammatory response that is usually accompanied by angiogenesis, fibrosis, scarring and anatomical distortion that can result in pain and infertility[3-5]. The consistent presence of fibrosis and myofibroblasts in endometriotic lesions also plays an important role during the pathogenesis of this disease[222, 223]. Because of these possibilities, model systems are critical to study endometriosis.

It is known that endometriosis is a disease that occurs spontaneously in humans and non-human primates[48, 224]. Due to ethical reasons, experimental animal models have been developed using primarily rodents and non-human primates to study the etiology and mechanisms of endometriosis[224]. Mice are one of the most suitable models due to the accessible cost, they are easily bred, they can be genetically manipulated and their reproductive system is well understood[225].

In this study, we used a mouse model of endometriosis (uterine transplantation into the peritoneal cavity) that develops lesions that have structures like what is observed in humans. This has been characterized by gene expression using qPCR and immunohistochemistry, which shows that there are similarities between mouse and human lesions[226]. We have performed RNA-seq to generate a comprehensive gene expression profile of mouse lesions compared with the mouse uterus.

The aim of this study is to further characterize these lesions and determine which pathways and genes are altered in endometriotic lesions. A whole transcriptome profile

(or RNA-seq) on 1-month lesions compared to control endometrium from the mouse model was performed. We wanted to identify the genes that are differentially expressed in the endometriotic lesions compared with the uterus of sham controls.

Based on our understanding, we hypothesized an increase in different inflammatory pathways and increase in fibrosis linked with collagen deposition

2.2 Materials and Methods

2.2.1 Induction of endometriosis in mice and sham surgeries.

Animals were maintained in a designated animal care facility according to the Michigan State University's Institutional Guidelines for the care and use of laboratory animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. Eight-week-old female mice with a conditional double fluorescent Cre reporter gene (Pgr $^{cre/+}$Rosa26 $^{mT/mG}$) were injected with estradiol (E2) (0.1µg/mouse) every 24 hours for 3 days, and then had either a surgical induction of endometriosis or a sham surgery (Figure 2.1A).

For animals undergoing induction of endometriosis (Figure 2.1C), endometriotic lesions were established by inoculating endometrial tissue into the peritoneal cavity. To access the peritoneal cavity, mice underwent a laparotomy under anesthesia and a midline abdominal incision (1 cm) was performed to expose the uterus in the female mice. One of the uterine horns was removed and placed in a petri dish containing sterile phosphate-buffered saline (PBS: pH 7.5). The uterine horn was opened longitudinally with scissors and then cut into small fragments (about 1 mm^3). The fragments were then suspended in 0.5 mL sterile PBS. The fragment suspension was injected back into the peritoneal cavity of the same mouse from which the uterus was taken for an autologous

transplantation. The abdominal incision and wound were closed with sutures and the skin was closed with surgical wound clips. Afterwards, the abdominal cavity was gently massaged to disperse the tissue around the peritoneal cavity. Ten days after surgery, wound clips were removed. After 1 month, the mice were euthanized as required by the Institutional Animal Care and Use Committee, the peritoneal cavity was opened, and endometriosis-like lesions were counted and removed under a fluorescence dissection microscope. The lesions and uterine tissues were fixed with 4% (wt/vol) paraformaldehyde for histological analysis and snap frozen for RNA.

A sham surgery group was included as a control (Figure 2.1B). The female mice undergoing sham surgery were inoculated with 0.5 mL of sterile PBS. To access the peritoneal cavity, mice underwent a laparotomy under anesthesia and a midline abdominal incision (1 cm) was performed to expose the uterus in the female mice. One of the uterine horns was removed and collected for future analysis. Following the removal of the horn, 0.5 mL sterile PBS was injected back into the peritoneal cavity of the same mouse. The abdominal incision and wound were closed with sutures and the skin was closed with surgical wound clips, respectively. After 3 months, the mice were euthanized, the peritoneal cavity was opened, and the remaining uterine horn was removed and fixed with 4% (wt/vol) paraformaldehyde for histological analysis and snap frozen for RNA.

2.2.2 RNA isolation

Total RNA was isolated from frozen tissue using TRIzol reagent (Life Technologies). RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). The extracted RNA was quantified using Nanodrop and Qubit©.

2.2.3 RNA-sequencing and data analysis

Sequencing was completed for 10 samples of total RNA from mice for Next - generation sequencing (NGS) library preparation and sequencing, submitted to the Research Technology Support Facility (RTSF) Genomics Core. Libraries were prepared using the Illumina Stranded mRNA Prep, ligation kit with IDT for Illumina RNA UD Indexes, following manufacturer's recommendations except that half volume reactions were performed. Utilizing a combination of Qubit dsDNA HS and Agilent 4200 TapeStation HS DNA1000 assays, the complete libraries were checked for quality control and quantified. The libraries were pooled in equimolar amounts and the pool quantified using the Invitrogen Collibri Quantification qPCR kit. The library pool was combined with other pools of Illumina Stranded mRNA libraries prepared by the Genomics Core to make use of a shared S4 lane. 20% share of the lane was performed, with a target output of 400 million read pairs. The combined pool was loaded onto one lane of an Illumina S4 flow cell and sequencing was performed in a 2x150bp paired end format using a NovaSeq v1.5, 300 cycle reagent kit. Base calling was done by Illumina Real Time Analysis (RTA) v3.4.4 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.20.0.

Reads overlapping Ensembl annotations (version 107)[227] were quantified with featureCounts (version 2)[228] prior to model-based differential expression (DE) analysis using the edgeR-robust method (version 3.40.2)[229]. The principal component analysis (PCA) plot used to verify group separation prior to statistical analysis was generated with the plotMDS function of edgeR. Principal component analysis of normalized counts was conducted with the prcomp function in R.

2.2.4 Identification of pathways using PANTHER database// Proteome analysis

FilterByExpr from edgeR was used to identify the genes expressed in mouse tissue. We used PANTHER (Protein Analysis Through Evolutionary Relationships) [230, 231] data base version17.0 classification system (http://www.pantherdb.org) to perform the overrepresentation analysis. Gene ontology (GO) analysis of the genes expressed in mouse tissue was performed for PANTHER GO-slim terms, Molecular function and Biological process. The PANTHER database version17.0 was also used to identify pathways by Reactome.

2.2.5 Ingenuity Pathway Analysis (IPA)

The genes that were identified as significantly upregulated or downregulated when comparing uterus and lesions, were used for network and gene ontology analysis. The data sets were uploaded into QIAGEN Ingenuity Pathway Analysis (QIAGEN IPA). The IPA software searches the Ingenuity Pathway knowledge base on interactions observed in the literature, between the genes of interest and genes already contained in the Ingenuity Pathway Knowledge Base generating gene networks and pathways. The differentially expressed genes were classified with canonical pathways.

2.2.6 Masson's Trichrome staining

Masson trichrome staining was used for the detection of collagen fibers in tissues and to visualize the tissue structure. Paraffin-embedded tissue sections were deparaffinized in xylene and rinsed in absolute alcohol series. After a water rinse, the sections were immersed in Bouin's Fluid overnight at room temperature. Then, the tissue sections were stained using the Trichrome One-Step Blue& Red Satin Kit Procedure (#KTTTRBPT StatLab/American Master Tech) following the manufacturer's instructions.

The kit steps included the used of Modified Mayer's Hematoxylin and One step Trichome Stain followed by the dehydration of the tissue in absolute alcohol series and final step of xylenes before cover slipping the tissue.

2.3 Results

2.3.1. Development of the Mouse Model of Endometriosis with a Double-Fluorescent Cre Reporter, Pgr $^{cre/+}$Rosa26$^{mT/mG}$

In our studies, we utilized a mouse model of endometriosis that uses mT/mG reporters, *Pgr $^{cre/+}$Rosa26$^{mT/mG}$* [129]. This mouse model was selected due to the easy identification of lesions differentiating them from the surrounding tissue and adjacent organs. *Pgr $^{cre/+}$Rosa26$^{mT/mG}$* mice have the unique characteristic of expressing green fluorescent protein GFP (mG) in Pgr-positive uterine cells while Pgr-negative cells express red fluorescent Tomato (mT).

Using this model (*Pgr $^{cre/+}$Rosa26$^{mT/mG}$*), two groups of animals (n=5) went through 3 days of E2 treatment prior to surgery (Figure 2.1A). We performed sham surgery, removing one of the uterine horns from the mouse and inoculating PBS into the peritoneal cavity (Figure 2.1B).

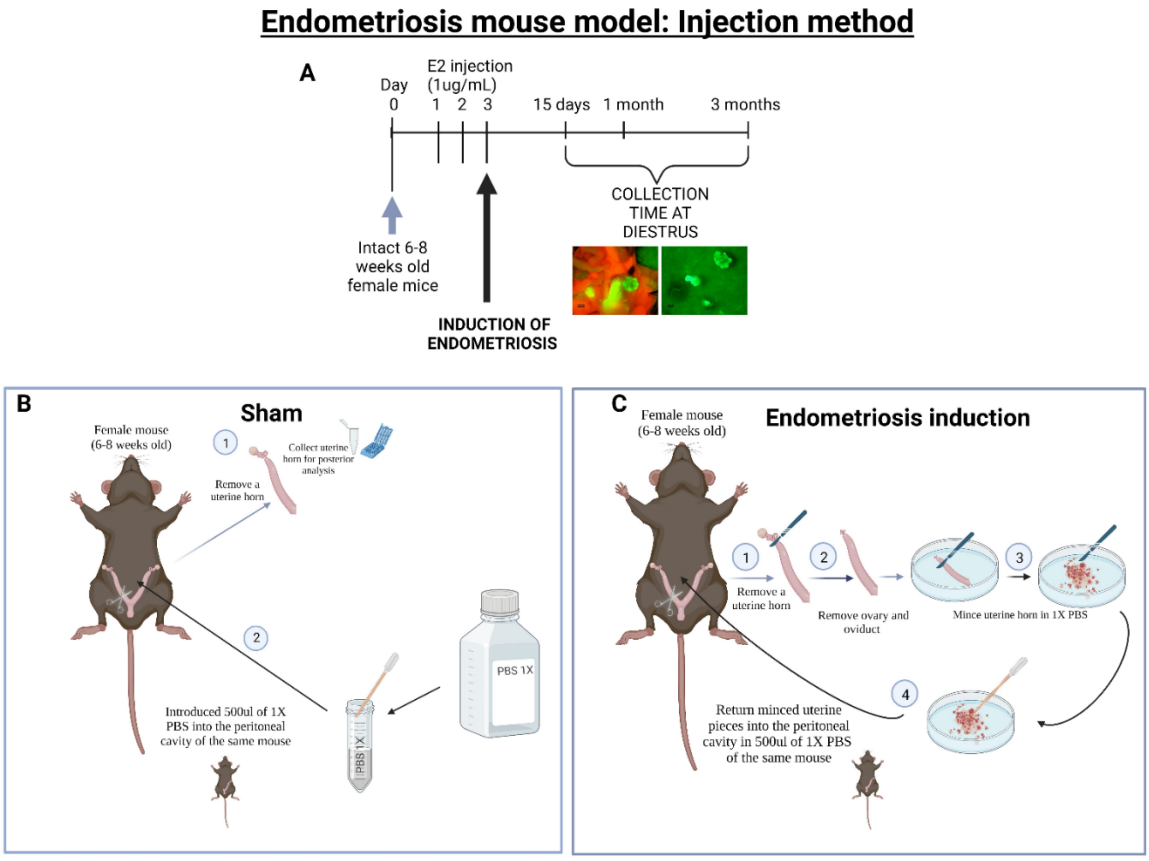

Figure 2.1. Endometriosis mouse model using injection method. (A) Schematic diagram showing the study design of endometriosis in female mice and the collection times of uterus or lesions of the double-fluorescence Cre reporter (*Pgr* $^{cre/+}$*Rosa26*$^{mT/mG}$) mice at diestrus for data collection. (B) Diagram showing the surgical procedure for sham surgery. (C) Diagram showing the surgical procedure of endometriosis induction surgery. Figure created with BioRender.com

In addition to this, we surgically induced endometriosis in a different set of mice. In this case, we removed one of the uterine horns and inoculated autologous endometrial tissue fragments (from the horn removed) into the peritoneal cavity (Figure 2.1C). Sham animals were collected at 3 months, using for the analysis the uterine horn left at the time of the surgery and as expected, no lesions were found during the collection time under the fluorescence dissection microscope. Endometriotic lesions were collected one month after surgery from the animals that went under endometriosis induction. Both surgical animal groups were collected at diestrus. The lesions collected from the mouse model

displayed similarities to those lesions recovered from endometriosis patients undergoing excision of endometriotic lesions. In both cases, the lesions showed similar epithelial and stromal compartments (Figure 2.2).

Endometriotic implants are often associated with fibrosis and structural distortion of adjacent structures and anatomy that can result in pain[232]. We performed Masson's trichrome staining to show the histological similarities between human and 3-month mouse endometriotic lesions and the resemblances of fibrosis in both species (Figure 2.2). Cytoplasm, muscle, intercellular fibers are stained red and collagen and mucus in blue.

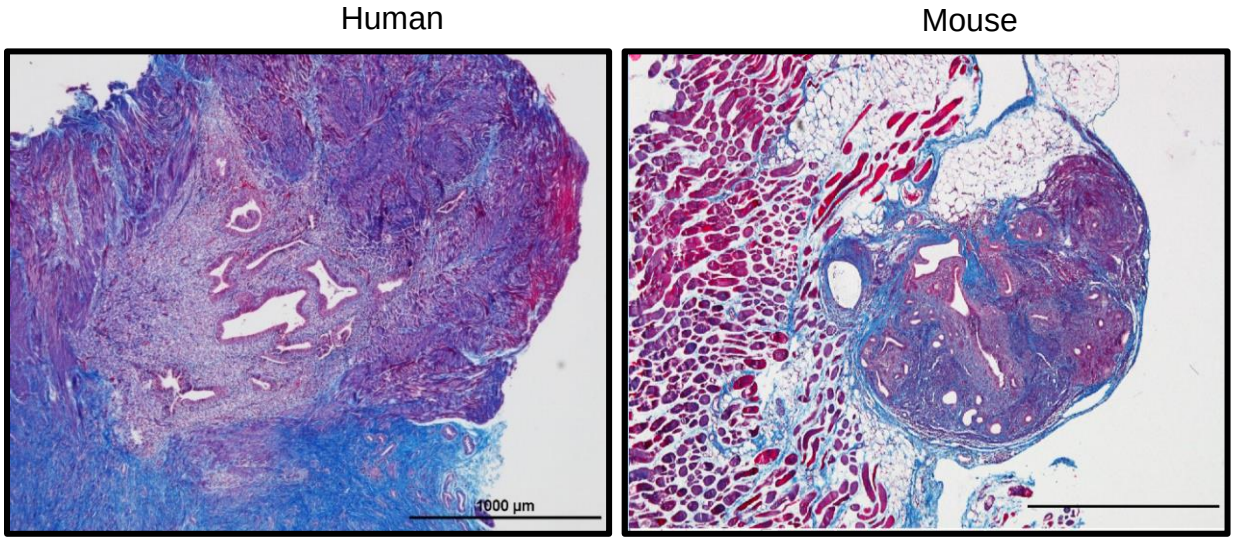

Figure 2.2. Human and mouse lesions. Representative histological similarities with Masson trichrome staining between endometriotic human lesions (left) and a 3-month mouse lesions (right). In both lesions, mouse, and human, we can observe the stroma and glandular epithelium. Blue area represents the deposition of collagen around the lesion. Scale bar = 1000µm

2.3.2. RNA-sequencing and edgeR analysis.

Seeking to characterize the transcriptome of the genes that are differentially expressed in endometriotic lesions compared with sham animals, we performed RNA-

sequencing analysis. A sequencing edgeR analysis was completed which determines whether there is evidence of significantly different genes across experimental conditions.

The samples were separated by principal component analysis (PCA) across PC1(69%) and showed a broad transcriptome of differences between endometrium and lesions (Figure 2.3A). From the RNA-seq results, we observed a total of 20,126 genes expressed. Of those 20,126 genes, 7,886 genes were differentially expressed (DEGs).

4,814 of those genes were found to be increased whereas 3,072 genes were found to be decreased (Figure 2.3B). Genes involved in (Figure 2.3C) connective tissue disorders (Supplementary table 2A), inflammatory diseases (Supplementary table 2B) and inflammatory responses (Supplementary table 2C), such as *Cd36*, *Cebpa* and *Lipa* were increased.

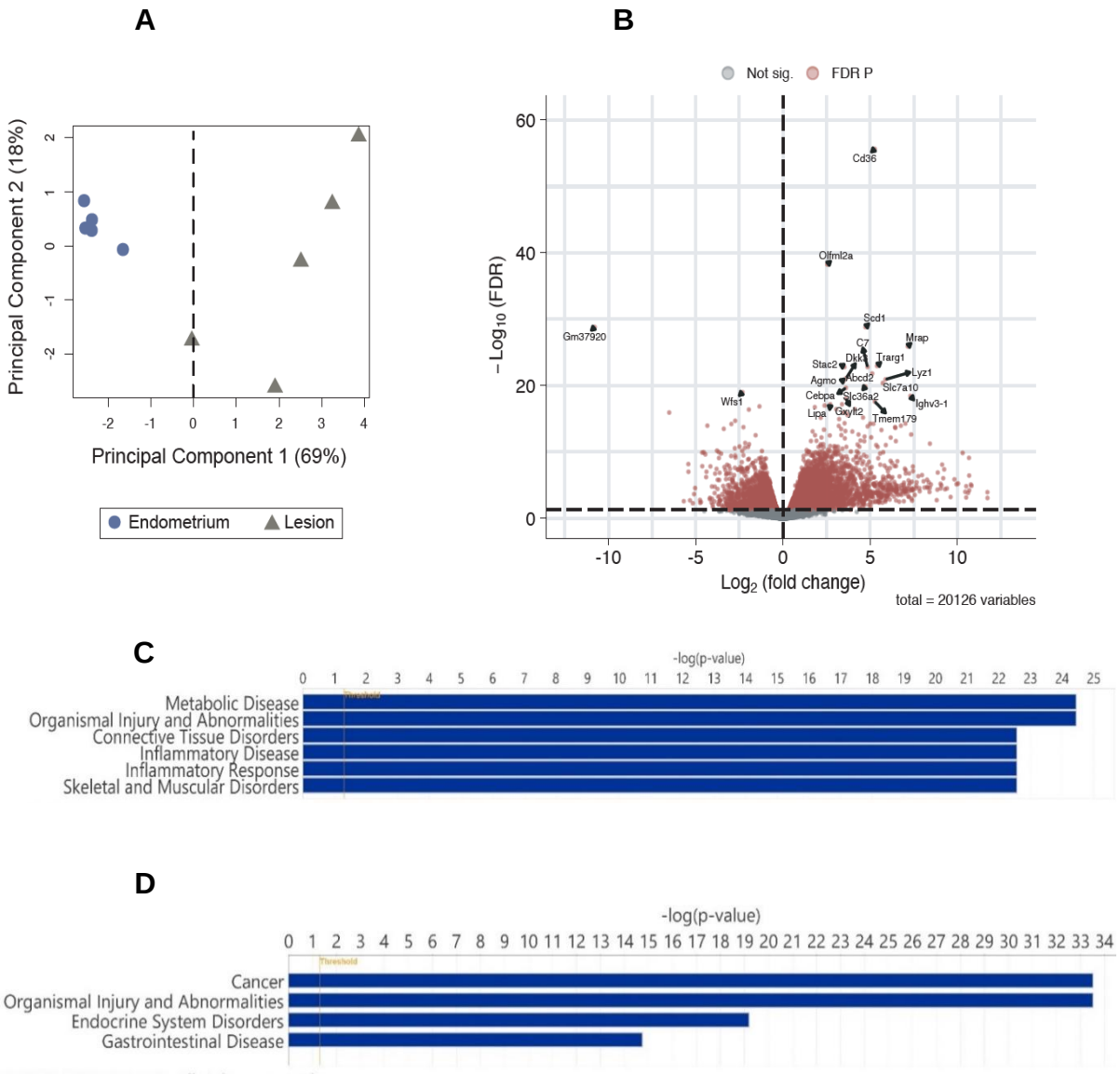

Figure 2.3. RNA-sequencing analysis of mouse lesions and mouse endometrium (A)RNA-sequencing results from lesions compared to sham uterus (n=10). The Principal Component Analysis (PCA) plot graphically shows transcriptome separation between endometrium and lesions. (B) Volcano Plot highlighting several top upregulated genes and downregulated genes in a total of 20,126 expressed genes. (C) The IPA top disease and disorders for upregulated and (D) downregulated genes.

Matrix metalloproteinases (MMPs) such as Mmp2, Mmp3, Mmp10 and Mmp13, and Col7A1, Col8A1, ColA12A1 were also upregulated (Supplementary table 2A ,2B and 2C) while genes involved in (Figure 2.3D) cancer and organismal injury (Supplementary table 2D) such as *Wfs1* were decreased.

We used PANTHER to analyze the function and the pathways involved in those differentially expressed genes between mouse lesions and mouse uterus. (Figure 2.4).

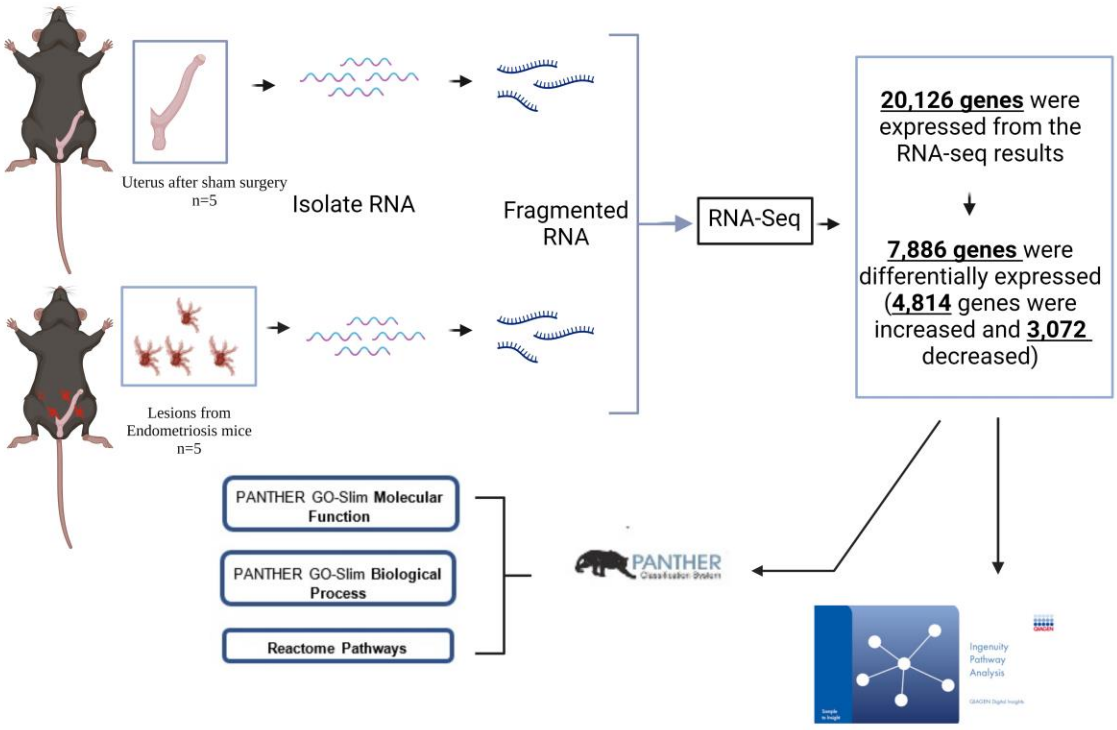

Figure 2.4. Comparison between sham surgery uterus and lesions from endometriosis mice. Number of genes identified after the RNA-sequencing with edgeR analysis. GO-slim selected to identify the role of the upregulated genes and downregulated genes. Figure created with BioRender.com.

2.3.3 PantherDB gene ontology analysis of mouse endometriotic lesions and sham uterus (Pathway analysis)

PANTHER is an integrated knowledgebase of evolutionary and functional relationships between protein-coding genes.[230, 233].

To delineate the mRNA contained in the mouse endometriotic lesions, we conducted PantherDB gene ontology (GO) analysis and introduce our findings separately

based on biological function (GO-Slim Biological Function), molecular function (GO-Slim Molecular Function) and pathways implicated (Reactome Pathways).

The enrichment of molecular functions of 1 month mouse lesions versus sham uterus from the increased genes revealed elevated (3.46-fold enrichment) Major Histocompatibility Complex (MHC) protein binding. The genes that were found decreased revealed a lower snoRNA binding activity (-4.03-fold enrichment) (Figure 2.5A).

Biological function analysis in increased genes showed an elevated B cell receptor signaling pathway (3.59-fold enrichment) and functions related to immunoglobin mediated immune response (3.35-fold enrichment). Other functions such as T cell activation, innate immune response, and inflammatory response were also elevated. The decreased genes showed a role in ribosomal subunit export from nucleus (-4.69-fold enrichment) and cytoplasmic translational initiation (-4.51-fold enrichment) (Figure 2.5B).

The Reactome pathway analysis in increased genes revealed that proteins implicated in PD-1 signaling (3.20-fold change), Antigen activates B Cell Receptor (BCR) leading to generation of second messengers (3.10-fold change) and Collagen chain trimerization (2.20-fold change) were increased. The decreased genes showed that proteins implicated in the cholesterol biosynthesis were considerably decreased (-4.84-fold enrichment) (Figure 2.5C).

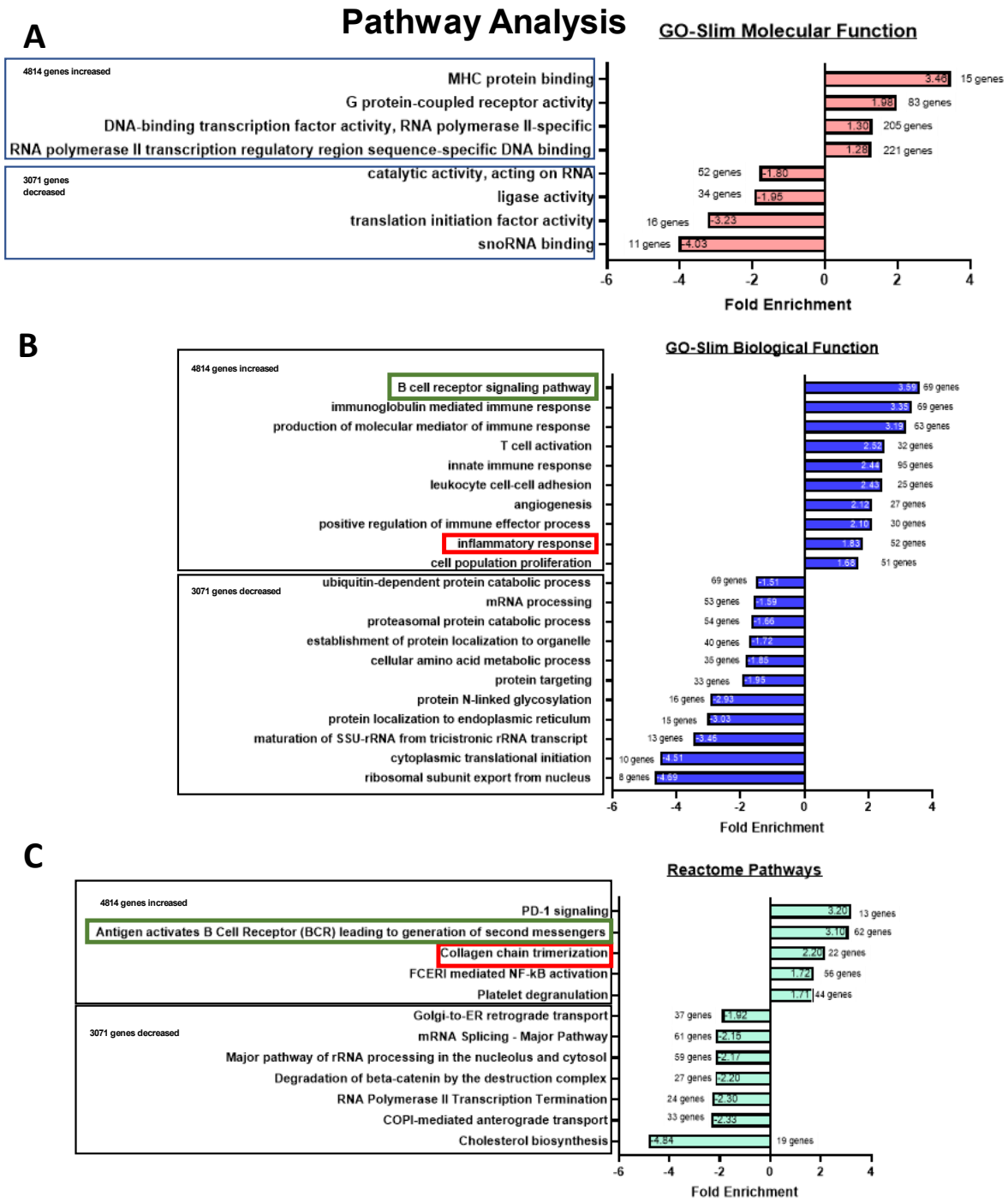

Figure 2.5. PantherDB gene ontology analysis between uterus (3 months) and mice lesions (1 month). (A) Molecular function and pathways implicated. (B) Biological process analysis and pathways implicated. Functions directly related to B cells (green box) and inflammation (red box) are enhanced. (C) Reactome Pathways. Functions directly related to B cells (green box) and collagen (red box) are enhanced. n=5 per group.

2.3.4. IPA Canonical pathways

Seeking to identify the genes that are differentially expressed in endometriotic lesions compared with sham animals, we performed RNA-sequencing analysis. Differential expression analysis revealed that 7,886 genes (4,514 increased, 3072 decreased) were significantly dysregulated (differentially expressed).

Ingenuity Pathway Analysis (IPA) of the increased gene set (4,514 increased) identified the most statistically significant overlaps with B cell Development, FcγRIIB signaling in B Lymphocytes, IL-15 signaling and PI3K signaling in B Lymphocytes (Figure 2.6A). On the decreased gene set (3,072 decreased) identified, the most statistically significant overlaps with pathways related to Cholesterol biosynthesis and tRNA charging (Figure 2.6B).

The Stacked Bar Chart (Figure 2.7A) displays the number of molecules in the increased gene set that are up-regulated (red). The chart shows the percentage of gene dysregulation within each specific pathway. For B cell development, a total 489 molecules which 123 were upregulated (25.2%), for FcγRIIB signaling in B Lymphocytes, a total of 533 molecules which 120 were upregulated (22.5%) and for IL-15 signaling, total of 528 molecules which 119 were upregulated (22.5%).

Figure 2.7B displays the number of molecules in the decreased gene set that are down-regulated (green). The chart shows the percentage of dysregulation within each specific pathway. For Super pathway of Cholesterol Biosynthesis, a total of 29 molecules which 17 were downregulated (58.6%), for tRNA Charging, a total of 39 molecules which 14 were downregulated (35.9%) and for other cholesterol biosynthesis pathways, a total 13 molecules per pathway which 9 were downregulated (69.2%).

Figure 2.6. Bubble Chart in Ingenuity Pathway Analysis of differentially expressed gene sets. (A) IPA Canonical Pathway Analysis of the 4814 increased genes from the 7886 genes differentially expressed between uterus and lesions (FDR<0.05). Showed the top 20 Canonical Pathways most significant enriched pathways (p<0.05). (B) IPA Canonical Pathway Analysis of the 3072 decreased genes from the 7886 genes differentially expressed between uterus and lesions (FDR<0.05). Showed the top 20 Canonical Pathways most significant enriched pathways (p<0.05). The plots were created in IPA.

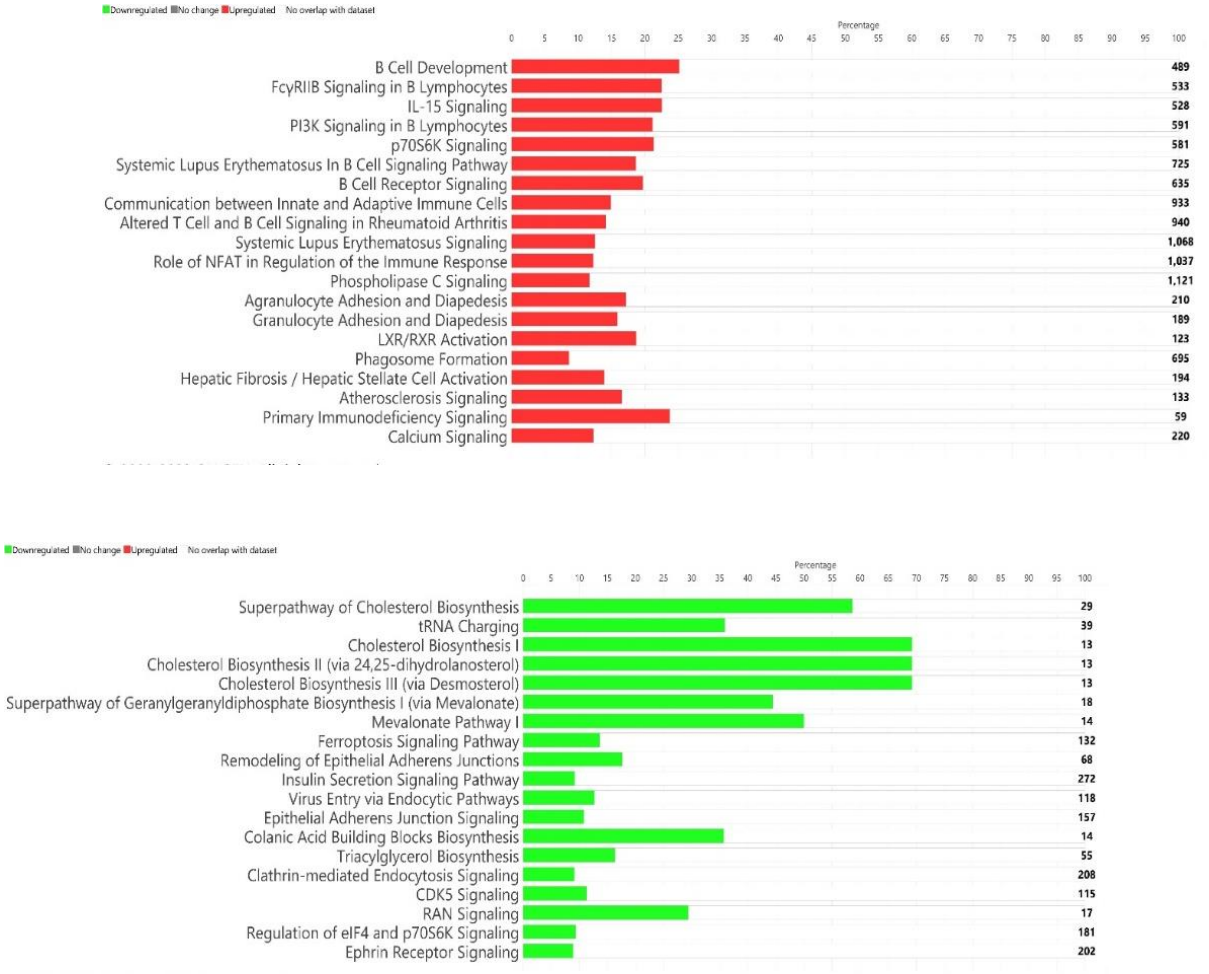

Figure 2.7. Stacked Bar Chart in Ingenuity Pathway Analysis of differentially expressed gene sets. (A) Total number of molecules involved for each Canonical pathway in the increased gene set (4,514 increased) (B). Total number of molecules involved for each Canonical pathway in the decreased gene set (3,072 decreased).

2.4 Discussion

Chronic inflammation and fibrosis are inherent hallmarks of endometriosis[50, 234]. The involvement of immune cells plays an important role in the pathogenesis of endometriosis[235]. Peritoneal neutrophils and macrophages secrete biochemical factors that can promote endometriotic cell growth, invasion, and angiogenesis. In addition to

this, the imbalance of T cell subsets leads to aberrant cytokine secretion and inflammation that enhances the growth lesions[236].

The human endometrium is a highly dynamic tissue that cyclically sheds and regenerates under hormonal influence. The immune cells in the endometrium play a key role in the prevention of infections and to help with the establishment of pregnancy.

In our studies we observed the relevance of genes involved in B cell development. B cell populations have been studied within the context of endometriosis and produce cytokines such as IL-6, granulocyte-macrophage colony-stimulating factor and tumor necrosis factor, which are involved in chronic inflammatory diseases[237]. However, some studies have shown that endometrial B cells have different characteristics when compared with circulating B cells[238]. This may indicate an active involvement within the endometrial immune system, emphasizing the importance of understanding the role of B cells in the endometrium.

Our findings revealed that many of the biological functions upregulated in one-month lesions were related to the inflammatory response. These functions not only include the B cell receptor signaling pathway as the pathway with the most genes involved, but also biological functions such as T cell activation, innate immune response, angiogenesis, and cell proliferation. Previous studies have shown in the pathogenesis of endometriosis that these cells may contribute to the disease secreting autoantibodies[239, 240].

In women with endometriosis, the peritoneal fluid is remarkable for an increased number of activated macrophages and importance of different cytokine present[241]. Activated B cells can produce cytokines such as IL-6, il-17 and IFN-γ, which also

participate in the pathogenesis of endometriosis[83, 100]. The increased number of genes upregulated related to B cells that we observed could suggest that they may be involved in the secretion of cytokines promoting the inflammatory state in the peritoneum during the development of endometriosis. In addition to this, results from this study regarding biological functions showed that B cell signaling pathways, immunoglobin mediated immune response, T cell activation and inflammatory responses are among the most relevant functions.

Furthermore, the analysis of the Molecular Functions showed the upregulation of the Major Histocompatibility Complex (MHC). The MHC is one of the most important components of immune dysregulation within endometriosis. It is also known as Human Leukocyte Antigens (HLA). It consists of cell surface proteins that mediate interactions between the immune responsive cells and plays a key role in the immune response which suggests that endometriosis shares many similarities with autoimmune diseases[242]. Abnormal expression of both Class I and II MHC antigens in endometriotic lesions, inhibits the cytotoxic activity of NK cells[242]. The inadequate removal of the eutopic endometrium can be a result of impaired antigen presentation by these tissues which can promote the development of the endometriotic implants[243]. NK cells are able to recognize HLA class I molecules on target tissues which abolishes their killing capability. Studies have shown a significant increase in HLA I and II in both glands and stroma in patients with endometriosis, compared to controls. This would indicate an increased resistance to NK cell lysis of endometrial cells in women with endometriosis[242]. Some studies have observed that patients with endometriosis has a significantly higher expression of HLA molecules compared to controls[244]. In our study, focused on mRNA

expression, we observed a similar trend. This suggest that endometriotic cells from women with endometriosis could be more resistant to the destruction of NK.

Transcriptomic analysis of endometrium of women with endometriosis has shown upregulation of some pathways involved in lymphocyte activation, cytokine induction and inflammation[245]. Quantitative analysis of gene expression is crucial for understanding molecular mechanisms that underline genome regulation. In this study we showed differentially expressed genes between mouse uterus and 1-month mouse lesions from mice with endometriosis. The overrepresentation analysis only considered the differentially expressed genes to study the different molecular and biological functions and the different reactome pathways. Genes associated with processes related to B cell receptor signaling, immune response, T cell activation, angiogenesis, inflammatory response, and cell proliferation were enriched.

In addition to the inflammatory component present during the development of endometriosis, other studies have showed that endometriotic implants are often associated with distortion of adjacent structures and scarring which is associated with fibrosis[5].

In this study, we have shown that genes such as Col7A1, Col8A1, ColA12A1 among other collagens involved in collagen chain trimerization, are upregulated. In addition to this, our data also indicated that matrix metalloproteinases (MMPs) were found to be upregulated. The MMPs play an important role maintaining the homeostasis of the extracellular matrix and any dysregulation of its expression could lead to the disease[246]. Several studies have reported levels of MMPs being elevated in patients with endometriosis[247, 248] and they can play a key role in different processes within the

disease. In our mouse data, we observed the upregulation of Mmp2[247], Mmp3[249], Mmp10[250] and Mmp13[251] which there is evidence to play a significant role in promoting the development of endometriosis. This fibrotic component of the endometrium combined with the altered immune status in women with endometriosis contribute to infertility, pain and early pregnancy loss [234, 252, 253].

The MMP family includes calcium-dependent zinc-containing endopeptidases, some of which not only affect the process of cell invasion but also participate in other physiological and pathological processes, such as angiogenesis and fibrosis[246]. There are different studies that have shown a role for MMPs in fibrosis. Studies related to the effect of BPA on endometriosis have demonstrated that the exposure to BPA contributed to fibrosis by increasing the synthesis of collagen I and III and decreasing MMP2 and MMP14 expression [254]. In addition, another study reported that the deficiency of specific MMPs could cause fibrosis endometriotic lesions due to the reduction in collagen degradation[255]. On the other hand, there are other studies that have observed that increased matrix stiffness promoted not only collagen I synthesis, but also MMP1 and MMP14 expression[256]. These studies suggest that there may be a balance between collagen synthesis and degradation that may contribute to the development of fibrosis in endometriosis. MMPs play a crucial role in the formation of collagen, which is important for the gradual progression of fibrosis during endometriosis[246].

In addition to this, our results from the Reactome pathways, indicate that PD-1 signaling, which is involved in the process of cell apoptosis[257, 258], Antigen activates B Cell Receptor (BCR) leading to generation of second messengers and collagen chain trimerization are the pathways with the most upregulated genes involved.

There are different theories that have been trying to explain the development of fibrosis in endometriosis. Epithelial to mesenchymal transition, fibroblast-to myofibroblast trans differentiation or increased production of collagen are some of the most studied [3] but there are other components that should be considered, such as microRNAs, that play a key role in fibrosis in fibrotic diseases [259] but not yet studied in the field of endometriosis.

The analysis of gene expression in this study showed the contribution of the different expressed genes in disorders such as connective tissue disorders, organismal injury and abnormalities, endocrine system disorders, inflammatory disease, and inflammatory response. Genes involved in the development of endometriosis such as Cebpa[260] and Cd36[261] were identified increased in connective tissue disorders and inflammatory diseases while other genes such as Wfs1 were decreased. These results demonstrate significant dysregulation in genes involved in inflammation and fibrosis at one month within the disease.

Currently, there are no effective treatments to either cure endometriosis or provide remission of the disease[262]. The current therapy has three main aims: to reduce the pain, to increase the chances of pregnancy in women with infertility caused by the diseases and to delay the recurrence of endometriosis as long as possible[263].There is clinical evidence that shows that oral GnRH antagonists are effective in patients with endometriosis at reducing symptoms[264, 265] but at this time, surgery is the only choice available for managing advanced cases of endometriosis, which include the majority of patients suffering from this disease and specially for those women who pursue a successful pregnancy.

After discussing the results obtained from the RNA-seq associated to inflammation and fibrosis, in the next chapters, we will investigate which components could play a relevant role in the association with fibrosis and endometriosis, and if mechanisms related to inflammation could potentially regulate it.

CHAPTER 3: ASSOCIATION OF microRNA-21 AND FIBROSIS DURING THE PROGRESSION OF ENDOMETRIOSIS

3.1 Introduction

Endometriosis is a fibrotic condition defined as the presence of endometrial like gland and stroma at ectopic sites, primarily, in the peritoneal cavity[3]. The consistent presence of fibrosis and myofibroblast in endometriotic lesions plays a crucial role in the development of the disease[222, 223].

Myofibroblast are contractile non-muscles cells that are usually activated in response to tissue injury with the purpose of repairing lost or damaged extracellular matrix (ECM). Enhanced collagen secretion and the subsequent contraction during the scarring process, are part of the wound healing response which is critical to restore the tissue integrity[266]. Transforming Growth Factor (TGF)-β and mechanical stress, such as the stiffness of the scar tissue, seems to be critical to activate myofibroblasts[267]. The activation can occur when the α-smooth muscle isoform of actin (α-SMA) is expressed de novo and incorporated in stress fiber-like bundle which are crucial to promote the specific myofibroblast function of contracting the ECM[266].

TGF-β is upregulated and activated in fibrotic diseases inducing myofibroblast transdifferentiation while promoting matrix preservation[268]. Several studies have reported an increase of TGF-β in the peritoneal fluid, serum, the ectopic endometrium and peritoneal tissue of women with endometriosis compared to women without the disease[109, 117].

Some of the pro-fibrotic effects of TGF-β are mediated through upregulation of its downstream effector connective tissue growth factor (CTGF)[268]. CTGF plays an

important role during the development of fibrosis. Its function is mainly focused on tissue remodeling and fibrosis, including excess ECM synthesis in different fibrotic disorders[269].

This fibrotic process in endometriosis promotes adhesions and subsequent anatomical distortions which could lead to infertility in women who suffer from this disease[122]. Although there are several studies focused on fibrosis in endometriosis, the mechanisms underlying its fibrogenesis remain still unclear.

miRNAs are small non-coding RNAs that regulate mRNA expression and consequently protein synthesis. The human genome contains over 1,000 miRNAs[270], each with several predicted mRNA targets. Multiple studies have implicated miRNAs in pathologies such as cancer, diabetes, cardiovascular disorders and gynecological diseases including endometriosis[143, 271]. miRNAs can potentially regulate different cellular processes such as apoptosis, angiogenesis, cell survival, proliferation, migration and fibroproliferative responses, to name a few. All of these processes are essential systems in the pathophysiology of endometriosis[271].

Many miRNAs (profibrotic miRNAs) are associated with the regulation of the pathogenic processes leading to fibrosis, such as TGF-β signaling, ECM production and deposition and fibroblast proliferation [259]. TGF-β mediates gene expression via activation of the canonical Smad signaling pathway from transcriptional activation. There are miRNAs that can be regulated by TGF-β and TGF-β itself can be regulated by miRNAs. miR-21 (miR-21) is one of the miRNAs that was found to be upregulated in different fibrotic diseases and that appears to be connected to TGF- β signaling[259]. In

addition to this, there are studies that have shown a positive association between miRNA-21 and CTGF emphasizing the role of miR-21 during the development of fibrosis[272].

miR-21 plays an important role in different biological functions including cell proliferation, fibrosis, migration, apoptosis, invasion, inflammation[273] and it is involved in the key regulatory pathways. All these processes, in which miR-21 intervenes, play an important role in the development and progression of endometriosis. However, the involvement of miR-21 in this disease is still unclear.

Different miRNAs databases (https://www.targetscan.org and https://mirdb.org) predicted more than 380 target genes for miR-21. Some of its targets are relevant to fibrosis contributing to TGF-β signaling. One of those direct targets of miR-21 is the inhibitory Smad, SMAD7. Some miRNAs can promote fibrosis by impacting this target[274] including miR-21. The evidence for the role of miR-21 in fibrosis comes from different studies focused on mainly cardiac[275, 276] and pulmonary fibrosis[277-279].

There are several studies that have reported different miRNAs, including miR-21[147, 280, 281], to be altered in women with endometriosis[137, 138, 282]. Previous studies from our laboratory, using the baboon model, have identified miRNAs that were altered as a consequence of the induction of endometriosis, including miR-21[147].

In this study we proposed that the dysregulation of miR-21 in ectopic stromal cells can modulate TGF-β signaling in endometriotic lesions by amplifying components of TGF-β signaling resulting in fibrosis. To date, miR-21 has been reported to be associated with fibrosis in different diseases. However, it is unknown if it plays a role in the fibrogenic process within endometriosis.

The purpose of the present study was to elucidate whether miR-21 plays a role in the development of fibrosis during the progression of endometriosis and to investigate the potential mechanisms in which miR-21 regulates this pathological process.

3.2 Materials and Methods

3.2.1 Induction of endometriosis in the mouse model

Animals were maintained in a designated animal care facility according to the Michigan State University's Institutional Guidelines for the care and use of laboratory animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. We used the Pgr $^{cre/+}$ Rosa 26 $^{mT/mG}$ mouse model. This mouse has a double-fluorescent Cre reporter and has the ability to express membrane-targeted tandem dimer Tomato (mT) prior to Cre-mediated excision and membrane-targeted green fluorescent protein (mG) after excision (Figure 3.1A). Six to eight week-old mice were injected with estradiol (E2) (0.1µg/mouse) every 24 hours for 3 days, and then surgical induction of endometriosis (Figure 3.1B) was performed as previously described[129]. Endometriotic lesions were established by inoculating endometrial tissue into the peritoneal cavity. To access the peritoneal cavity, mice underwent a laparotomy under anesthesia and a midventral incision (1 cm) was performed to expose the uterus and intestine. The left uterine horn was removed and placed in a petri dish containing sterile PBS. The uterine horn was opened longitudinally and then cut into small fragments. The fragments suspended in 0.5 mL sterile PBS were injected into the peritoneal cavity of the same mouse from which the uterus was taken for an autologous implantation, and the abdominal cavity was gently massaged to disperse the tissue.

The abdominal incision and wound were closed with sutures and skin was closed with surgical wound clips, respectively. After a designated time (15 days, 1 month and 3 months) and during diestrus (Figure 3.1C), the mice were euthanized, and endometriosis-like lesions were removed using a fluorescence microscope and counted.

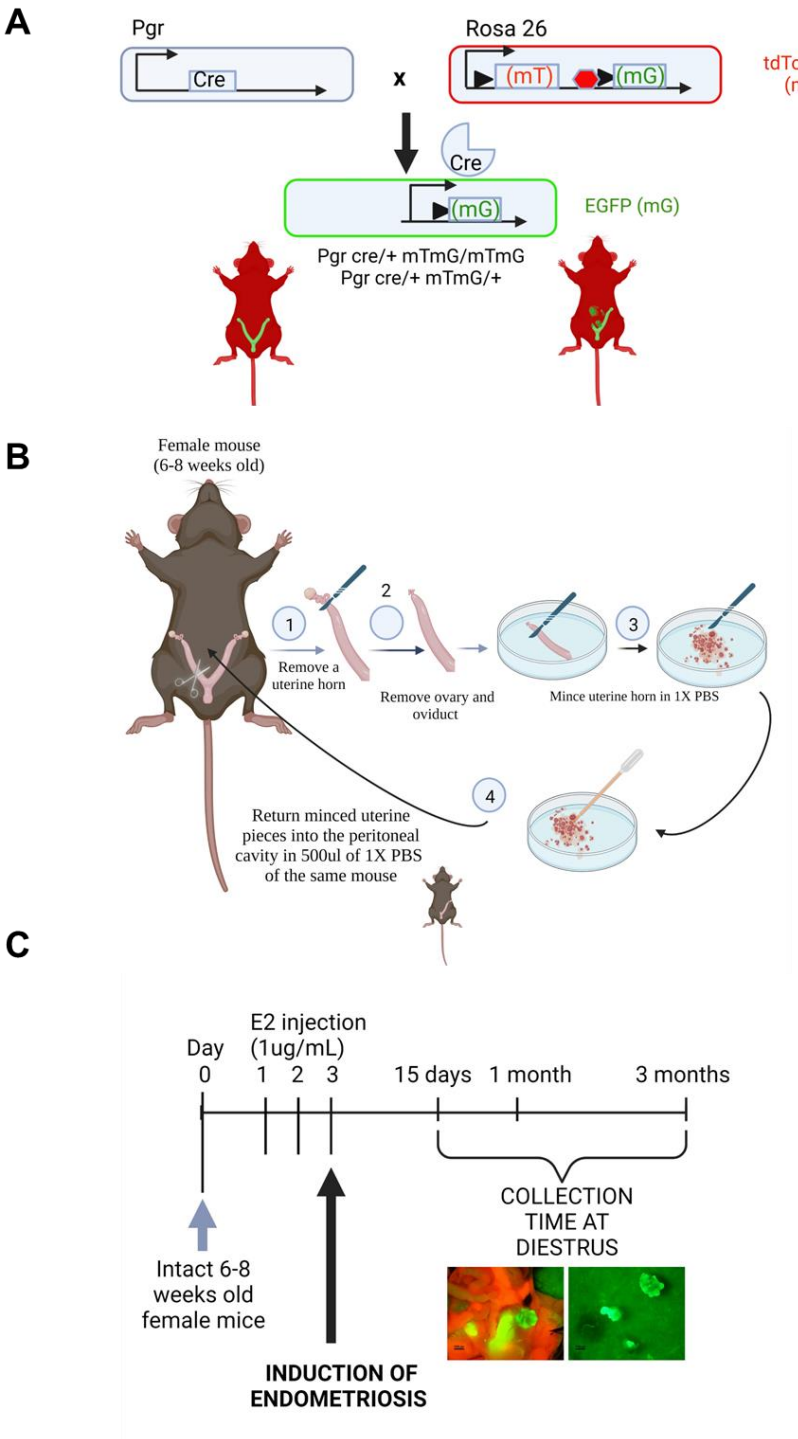

Figure 3.1: Mouse model used for the induction of endometriosis. (A) Schematic diagram of the mT/mG mouse construct before and after Cre-mediated recombination. (B)Diagram illustrating the surgical procedure for the induction of endometriosis using the double-fluorescent Cre reporter mouse (C)Schematic diagram of the induction of endometriosis in the mT/mG mouse model of endometriosis. Lesions for the analysis were collected at 15 days, 1 month and 3 months post induction during diestrus.

3.2.2 Baboon Endometriosis Model

All the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois, Chicago, and Michigan State University. Endometriosis was experimentally induced in female baboons (Papio anubis) by intraperitoneal inoculation (i.p) inoculation with menstrual tissue on two consecutive cycles, as previously described[48, 59, 283].

In the cycle before the induction of endometriosis, control eutopic endometrium (n=5) was obtained at laparotomy on day 10 post ovulation. Endometriosis was then induced in the same 5 animals by intraperitoneal inoculation of autologous menstrual tissue on 2 consecutive cycles. Following laparoscopic confirmation of endometriosis at the second inoculation, the animals (n = 5) were sampled at 3-month intervals post inoculation and euthanized at 15 months as required by the Institutional Animal Care and Use Committee, which permits a maximum of 4 invasive surgeries. At necropsy eutopic (EuE) and ectopic (EcE) endometrial tissues were collected and samples were snap-frozen in liquid nitrogen for RNA/protein extraction or fixed in 10% formalin for morphological and immunohistochemical analysis.

3.2.3 Human endometrial and Endometriotic Samples

Samples utilized for RNA analysis were obtained with Institutional Review Board (IRB) approval from the School of Medicine of the University of São Paulo. The patients who had a regular menstrual cycle and were primarily being treated for infertility were recruited. Other inclusion criteria were: (i) independent of symptoms, all the patients were subjected to transvaginal ultrasound with bowel preparation (TVUS-BP) evaluation; (we do not have the data of each patient’s symptoms) (ii) body mass index < 30 kg/m2; and

(iii) absence of other significant systemic diseases (e.g., hypertension and diabetes). Exclusion criteria were: (i) infection with HIV, hepatitis B or C, (ii) presence of abnormal vaginal bleeding, and (iii) consumption of illegal drugs or hormones. Informed consent was obtained from all patients. The patients suspected of having DIE, underwent laparoscopic resection of the ectopic tissue. Endometrial samples were collected from women with the endometriosis, before (EuE pre-op) and after the surgery (EuE post-op). Further, ectopic tissue (EcE) was collected during the surgical intervention. Endometrial samples were obtained with a Pipelle curette (Pipelle de Cornier, Laboratoire C. C. D., Paris, France) and stored in RNA later at −80 °C. Among the DIE group, we included 9 cases in which matched EuE (pre-op) and EcE could be collected during the mid-secretory phase.

For samples utilized for histology purposes, the study was reviewed and approved by the institutional review boards of Michigan State University and Spectrum Health Medical System (Grand Rapids, MI). Written informed consent was obtained from all human subjects. Human endometrial samples were obtained through the Michigan State University's Center for Women's Health Research Female Reproductive Tract Biorepository.

3.2.4 RNA Isolation and RT-qPCR

For the human and baboon samples, we utilized all 9 matched EuE and EcE from women with endometriosis and 5 matched EuE and EcE from baboons with induced endometriosis. Total RNA was isolated using the Trizol reagent (Life Technologies), and RNA concentration was checked using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). We performed TaqManTM assay for miR-21 expression analysis

and SYBRTM Green assay for CTGF using the ViiA7 qPCR System (Applied Biosystems). For the microRNA analysis, 100 ng of total RNA was reverse transcribed to cDNA using the TaqManTM MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed to assess the expression of miR-21 using the TaqManTM Universal Master Mix II with UNG (4440038, Applied Biosystems). The TaqManTM MicroRNA Assays (4427975, Applied Biosystems) with has-miR-21 (000397, Applied Biosystems) and U6 (001973, Applied Biosystems) snRNA were used for microRNA-specific RT-qPCR. For mRNA analysis, 1000 ng of total RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems). RT-qPCR was performed to assess the expression of the target gene expression using the PowerUpTM SYBRTM Green Master Mix (A25742, Applied Biosystems). The primer sequences for target genes analyzed using RT-qPCR are listed in Supplementary Table 3A . The expression data were normalized to U6 in the miRNA-specific RTqPCR and by RPL17 or 18S in the quantitative RT-qPCR. All quantitative reverse transcription-polymerase chain reactions were run for 40 cycles, and the fold change was calculated using the $2^{-\Delta\Delta Ct}$ method[284].

For mice samples, the procedure was the same as with human and baboon samples. Primers used for mice samples are listed in Supplementary Table 3A.

3.2.5 In situ hybridization (ISH)

For the mice tissues, we utilized 3 matched eutopic and ectopic tissue with induced endometriosis. Multiplex *in situ* hybridization assay for the co-detection of miR-21(FAM2X) and small nuclear 18S(bio2X) was performed as previously described[285]. Briefly, 4 µm formalin-fixed paraffin-embedded mice tissue was processed for the in-situ

hybridization assay on a Leica Bond Rx automated stainer. For the baboon samples, we utilized 3 matched EuE and EcE from baboons with induced endometriosis. miR-21 staining was calibrated by adjusting probe concentration and fluorescent substrate incorporation time so that no signal was detectable in adjacent normal tissue. Briefly, double-tagged miR-21 (FAM2X) and snRNA U6 (biotin2X) locked nucleic acid–modified DNA probes at 50 nmol/L each were hybridized to tissue slides. Expression of miR-21, U6, and cytokeratin (CK) 19 was assessed with appropriate antibody combinations, followed by sequential rounds of HRP-mediated deposition of appropriate fluorochrome-conjugated tyramine substrates for the baboon samples. Image-Pro Plus software version 7.0 (Media Cybernetics, Rockville, MD) was used for histogram-based image segmentation analysis. miR-21 signal intensity was scored in two locations, glandular epithelium and stroma, on a scale from 0 (no expression) to 3 (high expression). Whole-slide images were acquired using the Aperio Versa imaging system. PDAC tissues were utilized as quality control.

3.2.6 Histology and Immunohistochemistry

Tissues were fixed in 10% buffered formalin or 4% paraformaldehyde, embedded in paraffin, and sectioned at 6 μm thickness. Sections were then deparaffinized and rehydrated in a graded alcohol series. After antigen retrieval and hydrogen peroxide treatment (Antigen unmasking solution, H-3300, Vector Laboratories, Burlingame, CA), sections were blocked and then incubated with anti-CTGF (1:200 dilution, ab5097, abcam) and anti-Collagen I (1:1000 dilution, 1310-01, SouthernBiotech) overnight at 4°C. The next day, sections were incubated with biotinylated secondary antibodies followed by horseradish peroxidase conjugated streptavidin. Immunoreactivity was detected using

the DAB substrate kit (Vector Laboratories, Burlingame, CA) and visualized as brown staining.

3.2.7 Masson's Trichrome staining

Masson trichrome staining was used for the detection of collagen fibers in tissues and to visualize the tissue structure. Paraffin-embedded tissue sections were deparaffinized in xylenes and rinsed in absolute alcohol series. After a water rinse, the sections were immersed in Bouin's Fluid overnight at room temperature. Then, the tissue sections were stained using the Trichrome One-Step Blue& Red Satin Kit Procedure (#KTTTRBPT StatLab/American Master Tech) following the manufacturer's instructions. The kit steps included the use of Modified Mayer's Hematoxylin and One step Trichome Stain followed by the dehydration of the tissue in absolute alcohol series and final step of xylene before cover slipping the stained lesions.

3.2.8 Small RNA-sequencing

Total RNA was extracted from endometrium and endometriotic lesions from baboons with induced endometriosis for 15 months (n=8) or spontaneous disease (n=4). Small-RNA library preparation and high-throughput sequencing generated an average of 8.9 million reads per sample. Quality and adapter-trimmed reads (Trim Galore v0.3.3) were mapped to human miRNAs from miRbase (release 22) using the miRDeep2 (v0.0.7) pipeline. Differential expression analysis was conducted with edgeR (v3.22.5) using the edgeR-robust method.

3.2.9 Ingenuity Pathway Analysis (IPA)

The miRNAs that were identified as significantly dysregulated when comparing matching 15 months uterus and lesions, were uploaded into QIAGEN Ingenuity Pathway

Analysis (QIAGEN IPA). The Ingenuity Pathway Knowledge Base generated a network connecting fibrosis with the dysregulated miRNAs from the 15-month baboon samples.

3.2.10 Statistical Analysis

Data are shown as the mean ± standard deviation. We used Student's t-test to compare the means of the two groups and two-way analysis of variance (ANOVA) with Turkey's post hoc test least-significant difference was used for multiple comparisons. $P < 0.05$ was considered statistically significant (2-tailed). GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA) was used for data analysis.

3.3 Results

3.3.1 Small-RNA library and Ingenuity pathway Analysis (IPA)

To identify miRNAs that are differentially expressed, we performed small RNA-sequencing and found that a total of 586 miRNAs were expressed (≥ 2 CPM, ≥ 2 samples) in the induced model with 256 miRNAs (44%) differentially expressed (DE), ($q<0.05$) between matched ectopic and eutopic endometrium. When eutopic endometrium from the induced model was compared to spontaneous lesions, 233 of the 571 miRNAs were DE (41%), mirroring the differences identified in the induced model. A comparison of lesions from spontaneous versus induced endometriosis found 537 miRNAs with only 10 DE miRNAs (1.9%).

Based on the similarity of the induced and spontaneous disease, all samples were combined, and we found that 304 of 579 miRNAs were DE (52%) with 150 increased in the ectopic compared to the eutopic endometrium. Among the top DE miRNAs related to fibrosis, miR-21 was identified. The Ingenuity pathway analysis of the 304 DE miRNAs identified angiogenesis and organismal injury, including fibrosis, as the major functional

alterations in the predicted gene networks. Ingenuity pathway analysis of DE miRNAs identified enrichment for associations with endometriosis ($p=2.1x10^{-10}$) and activation of fibrosis ($p=1.2x10^{-9}$) (Figure 3.2).

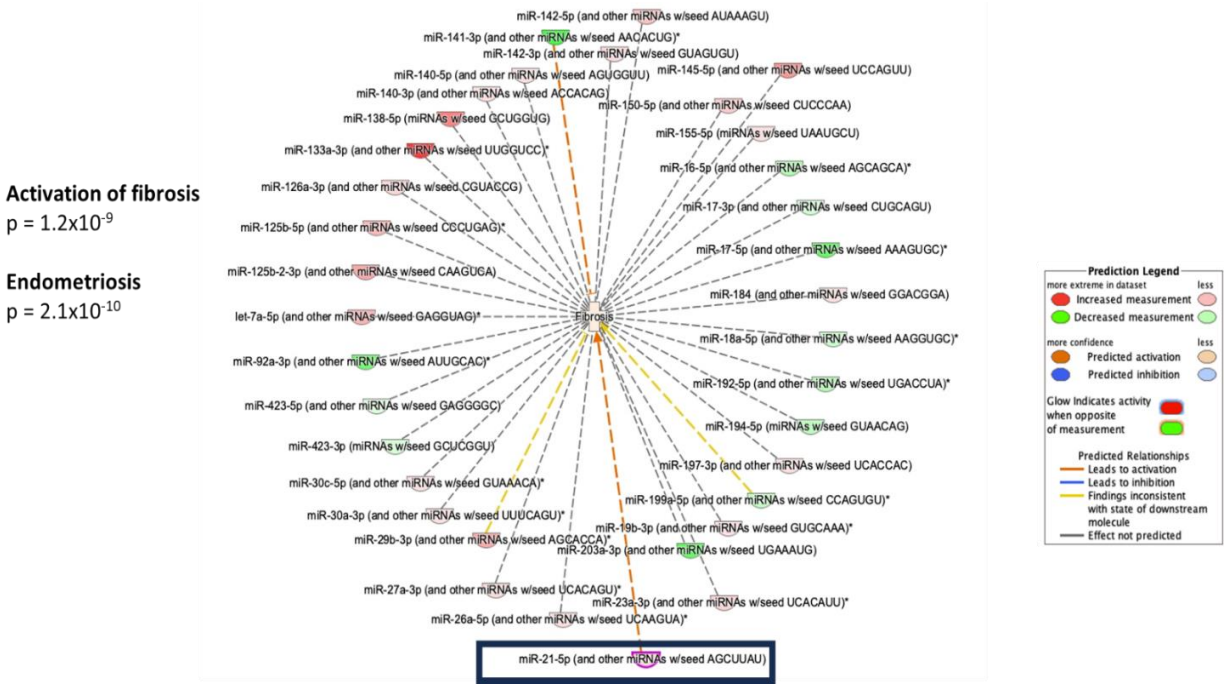

Figure 3.2: **Differentially expressed (DE) miRNAs associated with activation of fibrosis in endometriosis between eutopic and ectopic endometrium in 15-month baboon samples.** Small RNA-seq Analysis shows the most relevant miRNAs involved in the development of fibrosis including miR-21.

3.3.2 Progression of endometriosis in the mouse model.

To understand the progression of endometriosis and the differences overtime using the mouse model, we induced endometriosis in 6-8 weeks old female mice. Endometriotic lesions were collected at three different time points (15 days, 1 month and 3 months) during diestrus, after the endometriosis induction. Using the Double-fluorescence based on Cre-recombinase activity, Pgr $^{cre/+}$ Rosa $^{mT/mG}$, lesion sites could

be clearly visualized during the development of the disease and the number of lesions at each period of time counted (Figure 3.3A). Lesions around the peritoneal cavity were the sites where the most lesions were observed. After collecting the lesions, we observed that the number of lesions increased during the development of endometriosis showing a significant difference between one month and three months (Figure 3.3B). Related to the lesion weight, no differences were observed among the three different time points (Figure 3.3C).

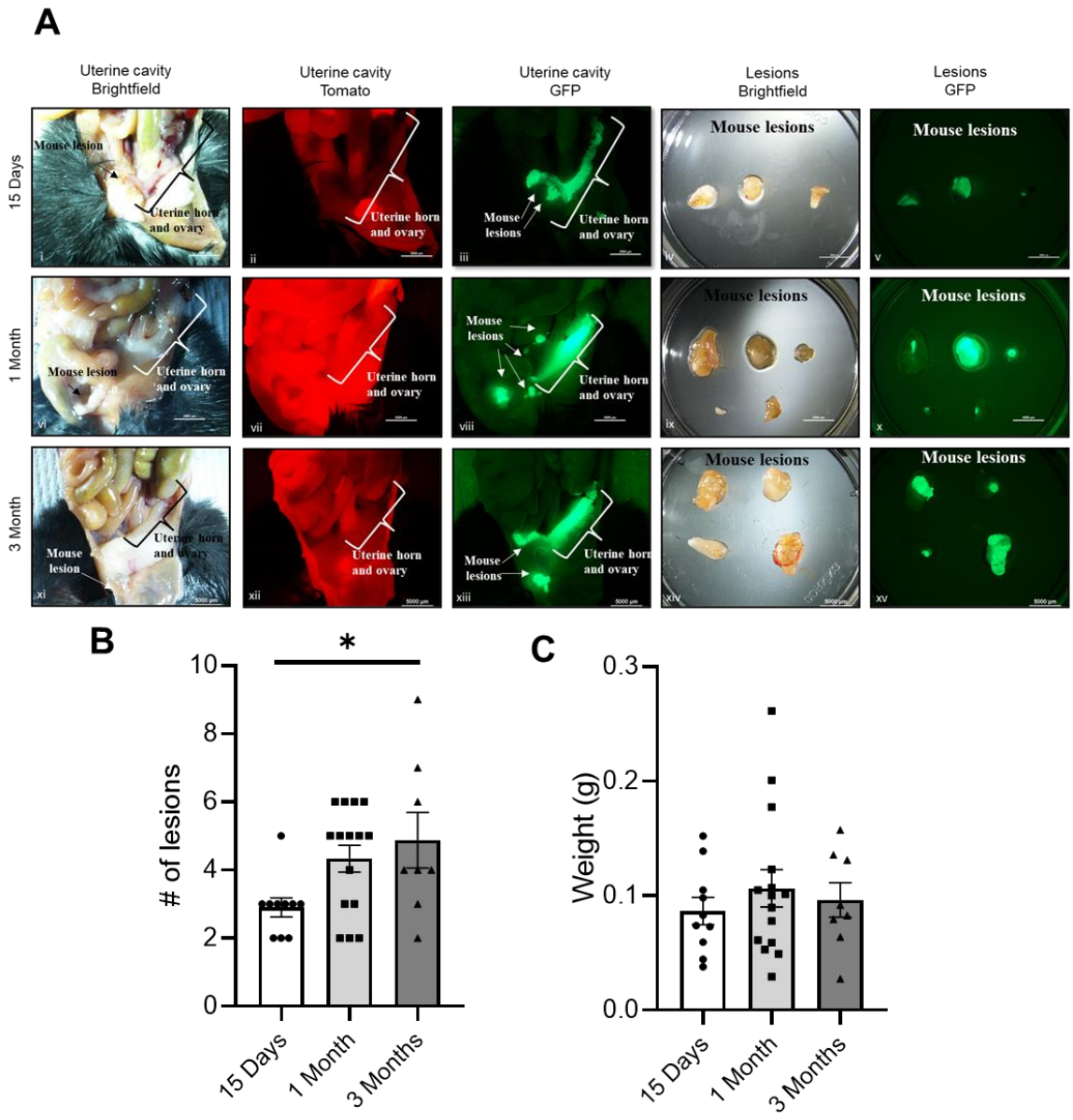

Figure 3.3: Progression of endometriosis using the Pgr $^{cre/+}$ Rosa $^{mT/mG.}$ mouse model. (A)Brightfield image of the uterine cavity of the mouse at 15 days, 1 month and 3 months after induction of endometriosis followed by uterine images showing the double fluorescence. Lesions collected from those animals are shown with brightfield and green fluorescence. Scale bar:5000µm. (B) Comparison of the numbers of lesions and (C) weight of the lesions overtime.

3.3.3 miR-21 is increased in endometriotic lesions

To characterize the expression of miR-21 in vivo in the mouse model, we first performed RT-qPCR on matched samples (uterus-lesion) at the different time points of collection (15 days-n=3, 1 month-n=6 and 3 months-n=6). The RT-qPCR analysis revealed that the expression of miR-21 was increased in lesions compared with the matched uteri of the mice and increased overtime up to 1 month. (Figure 3.4A).

In addition to this, we validated these results in baboons with induced disease (n=4). We observed a significant increase in miR-21 ectopic compared with the eutopic lesions at 15 months after endometriosis induction (Figure 3.4B), similar to the pattern of expression of miR-21 that was observed in the mice.

The same type of analysis was performed in women on matched mid-secretory eutopic and ectopic (lesions) from women with deep infiltrating endometriosis (DIE). We observed an increasing trend in the expression of miR-21 but this increase was not significant (Figure 3.4C). Our data suggests that miR-21 is increased in mouse, baboon and human lesions. The miRNA was statistically significant in the mouse and baboon over time.

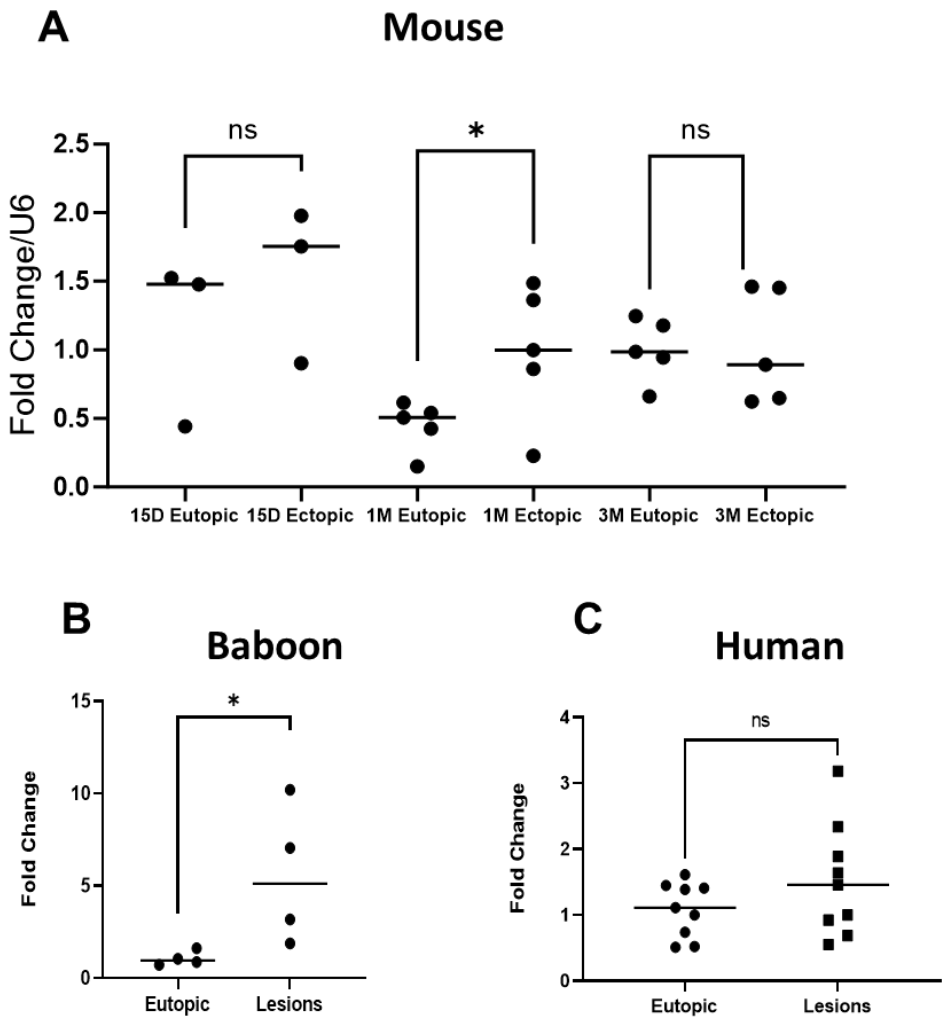

Figure 3.4: RT-qPCR analysis of miR-21 expression. (A) Expression of miR-21 in mice during 15 days, 1 month and 3 months (n=5) ,(B)baboon at 15 months after endometriosis induction (n=4), (C) and women (n=9)

3.3.4 miR-21 is predominantly expressed in the stroma of endometriotic lesions

To identify the cell-specific location of miR-21 in vivo, we next performed *in situ* hybridization on 3-month lesions from mice and matched mid-secretory eutopic and ectopic lesions in the baboon model of endometriosis. In the mouse model, the *in situ* hybridization analysis revealed that miR-21 was predominantly expressed in the stromal cells. (Figure 3.5). In figure 3.5 (first column), the expression of miR-21 in green in a

control uterus post endometriosis inoculation compared with three different lesions collected at three months. We observed the high expression of miR-21 in the lesions compared to the control uterus. The miR-21 cell class indicated a higher intensity of the miR-21 expression compared to the uterus surrounding the mouse lesion. In figure 3.5 (third column), we use 18SrRNA was used as a reference noncoding RNA for the uterus and the lesions. In the fourth column we can also observe the 18S rRNA cell class showing the location.

These findings were supported in computer assisted image analysis and figure composition in which cell class segmentation was performed based on the expression levels of DAPI, miR-21 and 18srRNA (Figure 3.6A). Figure 3.6A shows an example of a mouse lesion and the area in which miR-21 was intensified. In the upper left panel, in the first column, miR-21 is represented in green. In the second column, miR-21 cell class represents the intensity of the presence of miR-21 in a mouse lesion. Blue dots represent low miR-21 signal in the area selected, yellow represents low/medium intensity and red represents medium/high intensity of miR-21 within the area selected. In figure 3.6A, the left panel represents the 18srRNA control probe. In figure 3.6B, the left graph shows the quantification of the segmented distribution of miR-21 expression in the lesion, differentiating the expression between the glands and stroma. Figure 3.6B, the left graph shows the quantification of the segmented distribution of 18S rRNA expression in the lesion, differentiating the expression in glands and stroma as well.

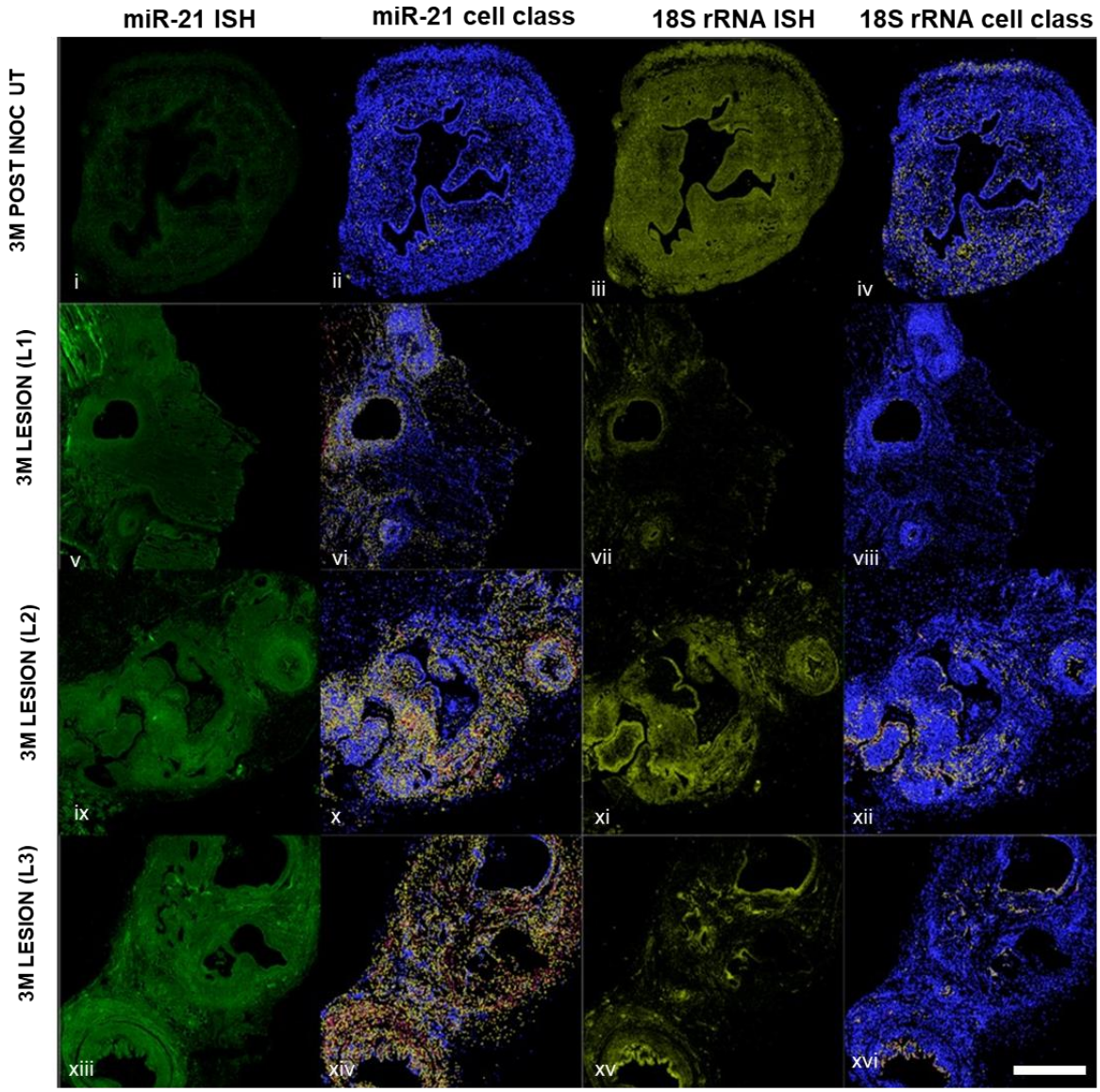

Figure 3.5: *In situ* hybridization of miR-21 expression and 18SrRNA expression in mouse uterus and lesions (n=3). Expression of miR-21 was higher in the stromal compartment of the endometriotic lesions compared (v,ix, xiii) with the uterus (i, ii). n=3 Scale bar:500μm

These findings were also validated in the baboon model (Figure 3.7). The *in situ* hybridization showed that miR-21 expression is predominantly in the stromal cells of the lesions. Figure 3.7 shows the detection of miR-21, cytokeratin and U6 in the eutopic endometrium of the baboon and the right panel shows the ectopic endometrium (lesion)

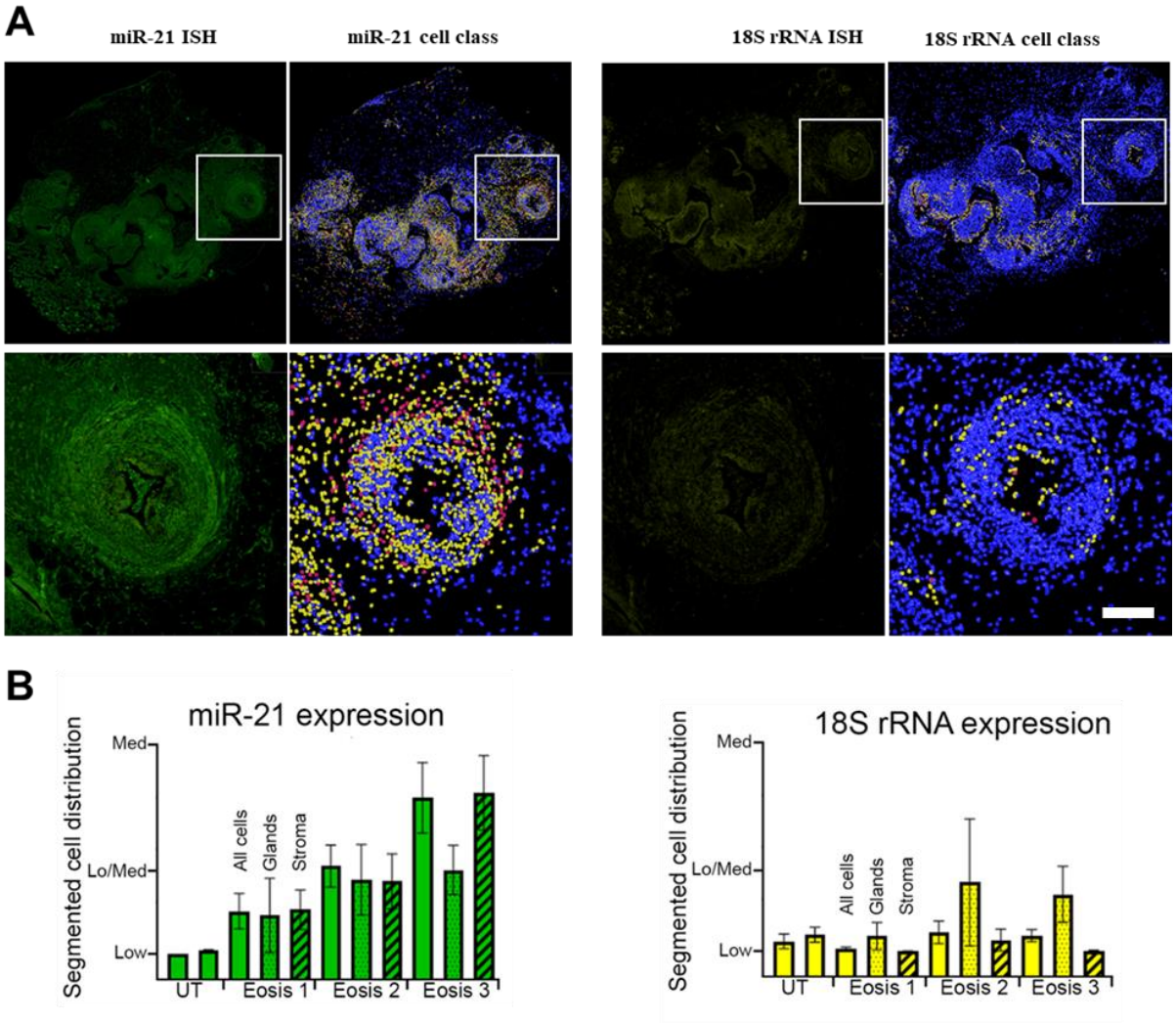

Figure 3.6: Computer assisted image analysis of segment cell distribution of miR-21 expression and 18S rRNA. miR-21 signal intensity was scored in two locations, glandular epithelium and stroma, on a scale from 0 (no expression) to 3 (high expression). n=3. Bottom Scale bar:200 µm. Upper scale bar: 500µm.

from the baboon. MiR-21 is represented in green, cytokeratin in pink and U6 in yellow. The two bottom pictures represent the co-detection of miR-21, the reference noncoding RNA U6 and cytokeratin. In these micrographs, DAPI signal was used as nuclear counterstaining.

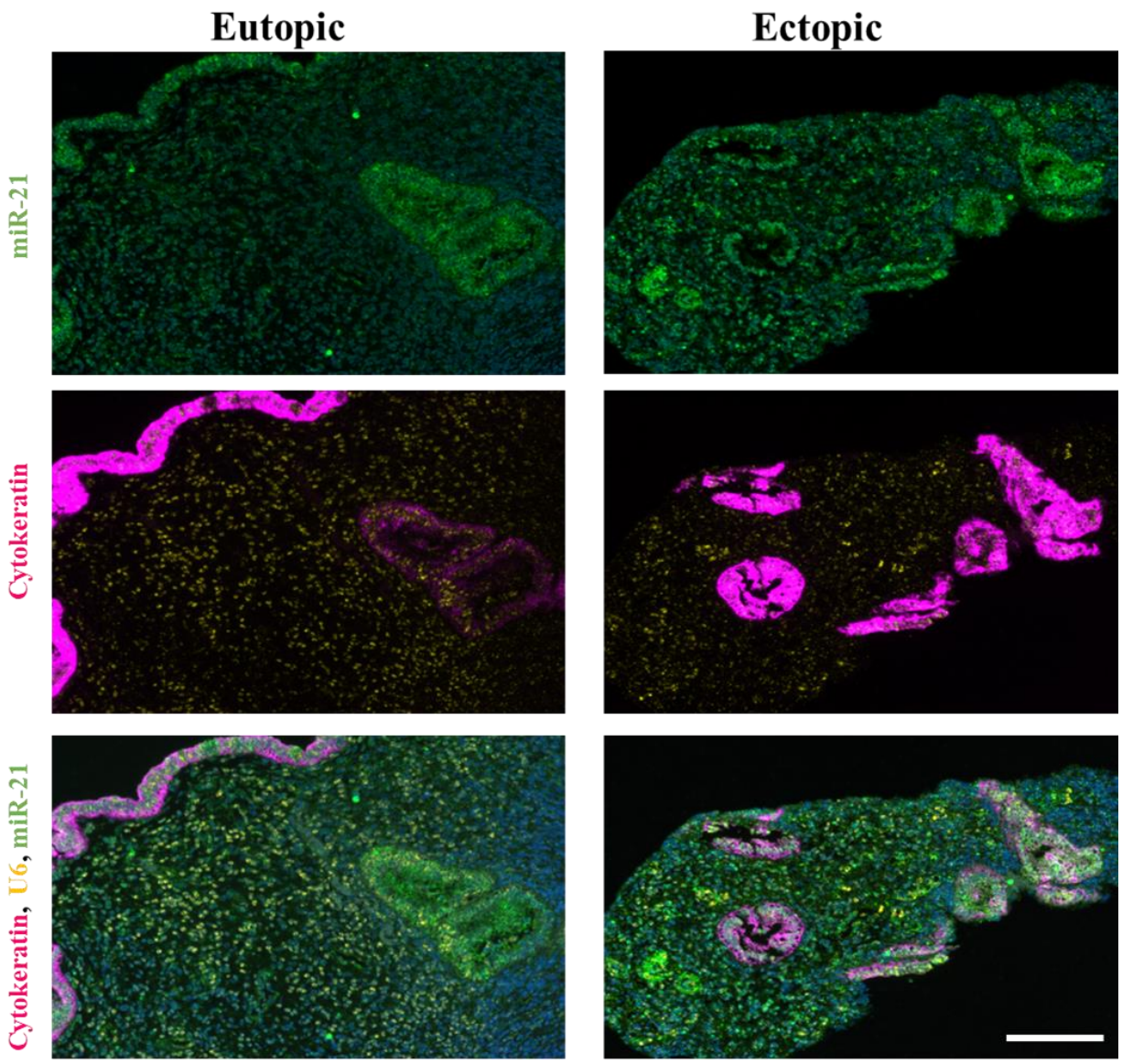

Figure 3.7: *In situ* hybridization of miR-21 in the baboon model. The left panel shows the *in situ* hybridization of the eutopic endometrium of the baboon and the right panel shows ectopic endometrium (lesion) of the baboon. miR-21 is represented in green, cytokeratin in pink and U6 in yellow. (n=3). Scale bar 300px.

3.3.5 miR-21 expression and fibrosis development

In order to determine the role of miR-21 during the development of fibrosis within endometriotic lesions, we used the *in situ* hybridization analysis of the 3 months mouse lesions (Figure 3.8A) and contrasted them with Masson's trichrome staining of the same lesions (Figure 3.8.B) . In the lesions, we observed that areas where collagen was accumulated (represented in blue) in the trichrome staining, matched with areas where miR-21 was present by *in situ* hybridization, suggesting a connection between the expression of miR-21 and the presence of collagen within the lesions.

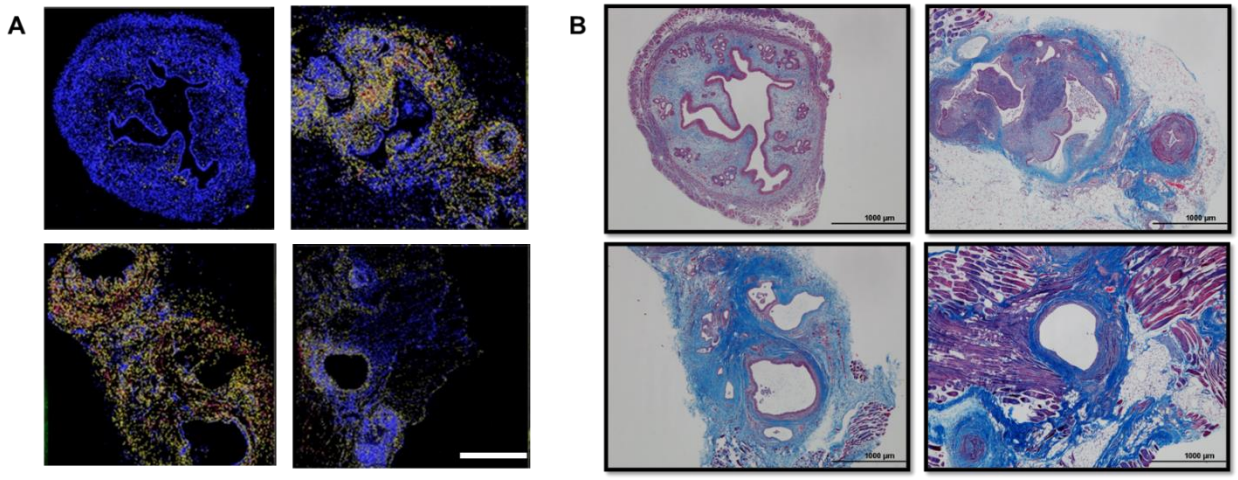

Figure 3.8: Correlation of miR-21 expression with fibrosis in mouse uterus and three months mouse lesions. (A) Segmented cells in *In situ* hybridization of miR-21 and 18S. miR-21 is present in the stromal cells surrounding the endometriotic lesions (B) Masson's trichrome staining of the mouse uterus and 3 month lesions. Deposition of collagen(blue) around the endometriotic lesion. (n=3 White and black Scale bar:1000µm).

3.3.6 Progression of fibrosis overtime in the mouse model

To gain more insight into the progression of fibrosis overtime in the mouse model, and to identify the cell-specific location of CTGF and Collagen I, we performed an IHC staining on lesions at different time points (15 days, 1 month and 3 months). We found that CTGF and Collagen I staining were mostly seen in the stromal compartment as endometriosis progresses (Figure 3.9). We also performed Masson trichrome staining to

quantify the extent of fibrosis in endometriotic lesions and compared them with the findings from IHC staining. The Masson trichrome staining revealed the deposition of collagen surrounding the lesion during the three time points.

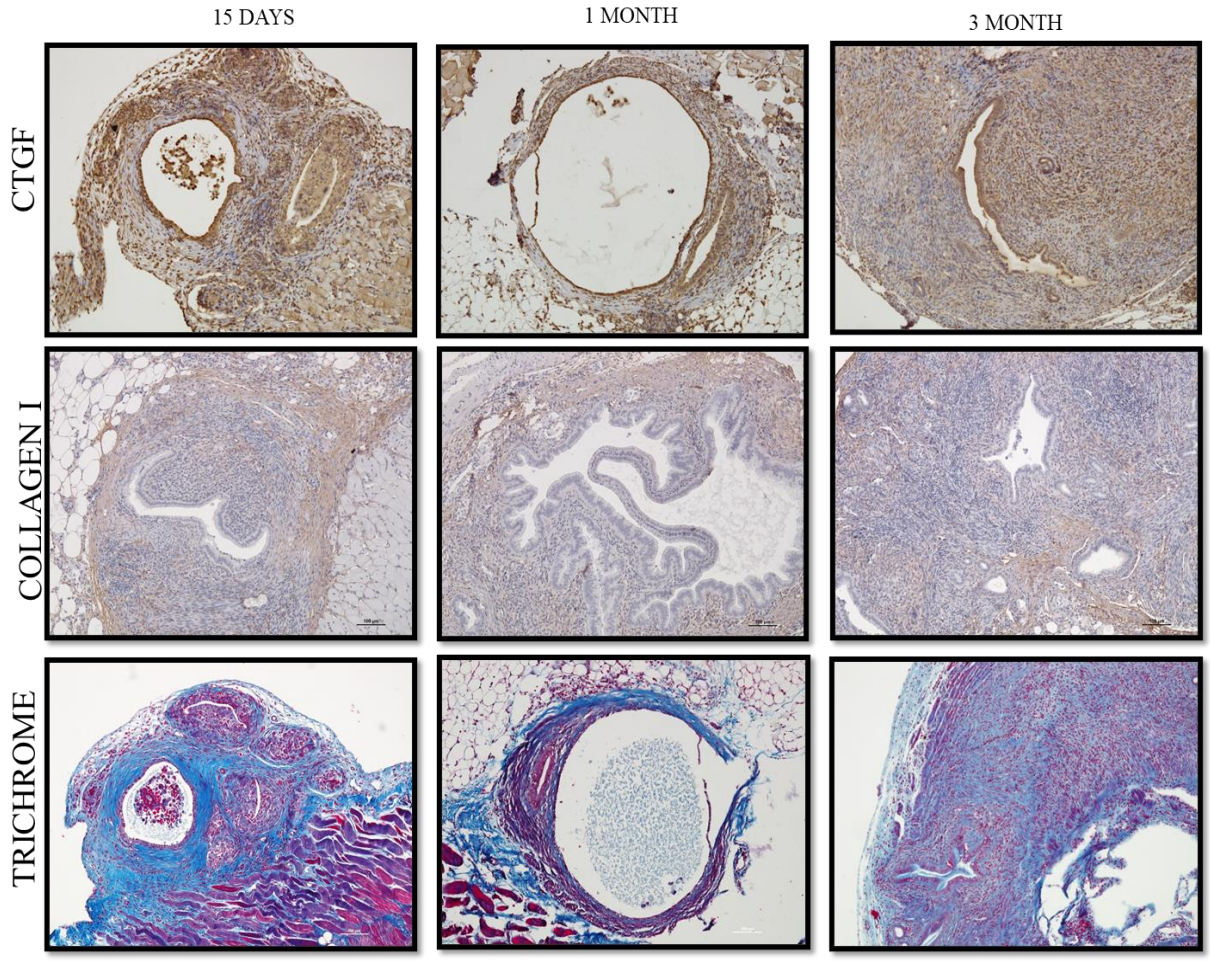

Figure 3.9: Representative sections of CTGF, Collagen I and Masson Trichrome staining in endometriotic lesions from the mouse model at 15 days, 1 month and 3 months after endometriosis induction. We observed the progression of fibrosis overtime and the intensity of the deposition of collagen by Masson's trichrome, n=4 per group Scale bar: 100μm

3.3.7 Presence of fibrosis overtime in the baboon model

In order to validate our findings from the mouse model in the baboon and confirm the presence of fibrosis overtime, we performed an IHC staining of CTGF and Collagen I in pre-inoculation endometrium, and the lesions stained at 15 months post-inoculation from 5 baboons (Figure 3.10). We observed that CTGF staining was not only strongly present in the 15 month lesions but also in the eutopic endometrium at 15 months. We observed a similar pattern with Collagen I, indicating an increase in Collagen I in 15 month lesions and in the eutopic endometrium. Masson trichrome staining confirmed an increase in the deposition of collagen in lesions at 15 months. These observations confirmed that the extent of fibrosis is progressively increased in endometriotic lesions and eutopic endometrium in baboons with endometriosis.

CTGF plays an important role during the development of fibrosis. In order to explore the characterization of CTGF in vivo and to complement the data obtained from immunohistochemistry, we performed RT-qPCR on matched samples, obtained at 15 days in mice, matched mid-secretory eutopic endometrium and ectopic endometrium (lesions) from baboons and women with endometriosis. The RT-qPCR analysis revealed that the expression of CTGF was significantly increased in the mouse model ($P<0.05$) (Figure 3.11A), baboon ($P< 0.001$) (Figure 3.11B) and human ($P< 0.001$) (Figure 3.11C).

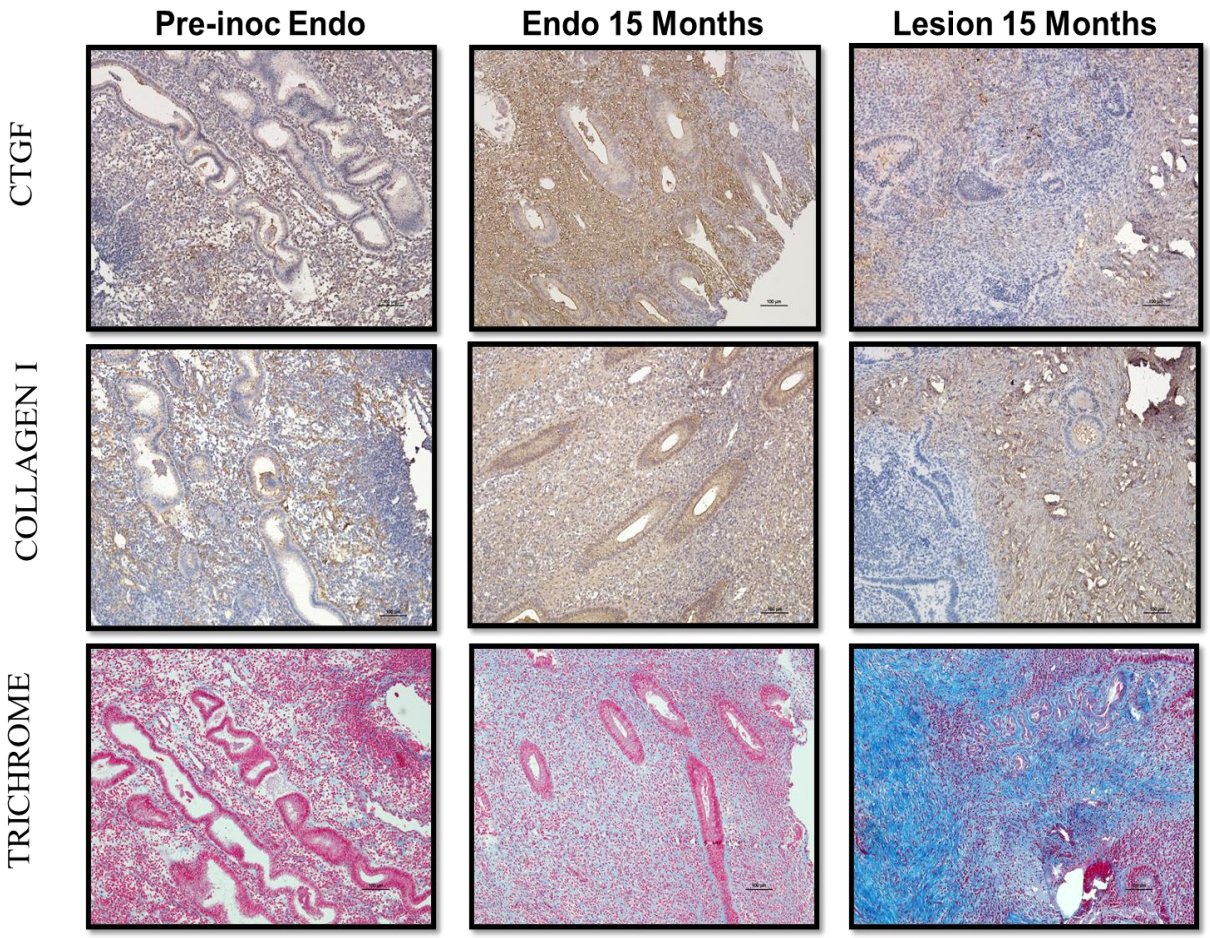

Figure 3.10: Representative sections of CTGF, Collagen I and Masson Trichrome staining of Pre-inoculation endometrium, Endometrium at 15 months post-induction and 15 months. We observed the progression of fibrosis over time 15 month time period. n=5 per group and Scale bar: 100μm

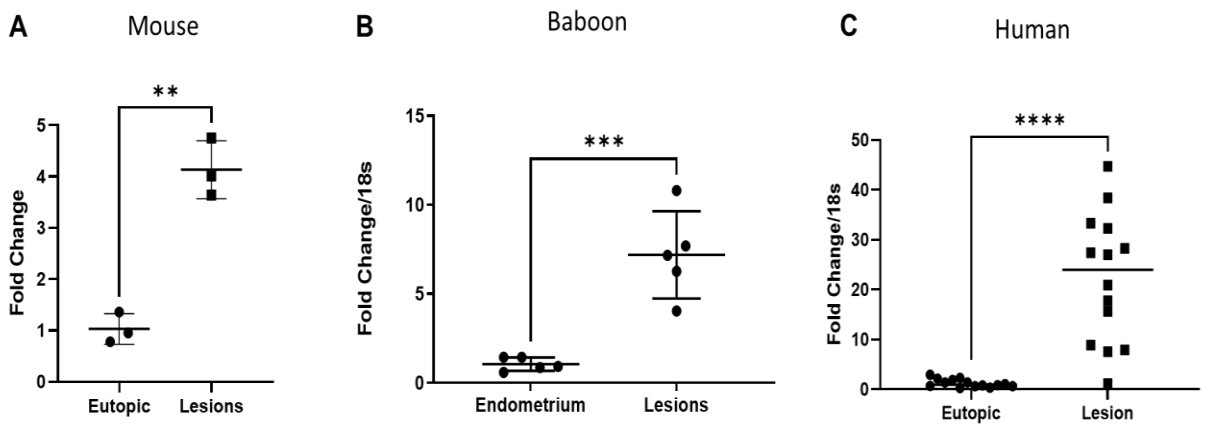

Figure 3.11: Expression of CTGF in mouse, baboon and women with endometriosis. A significant increase in CTGF expression in lesions was evident in all the species ($p<0.05$).

To confirm the severity of fibrosis in the baboon model, after 15 months of endometriosis induction, samples of adhesive tissue around the peritoneal were collected. To confirm the nature of the tissue, Masson trichrome staining was performed which confirmed not only the fibrotic nature of the tissue, but also the presence of lesions embedded into the fibrotic tissue (Figure 3.12). These observations confirmed the previous findings regarding the intensity and the instant of fibrosis at 15 months after the disease induction.

3.3.8. Presence of fibrosis and characteristics of human endometriotic lesions

To evaluate the incidence of fibrosis in women, we performed Masson trichrome staining in lesions from women with endometriosis (Figure 3.13). In this case, and due to the nature of the disease, it was not possible to determine when exactly the disease was stablished in these women.

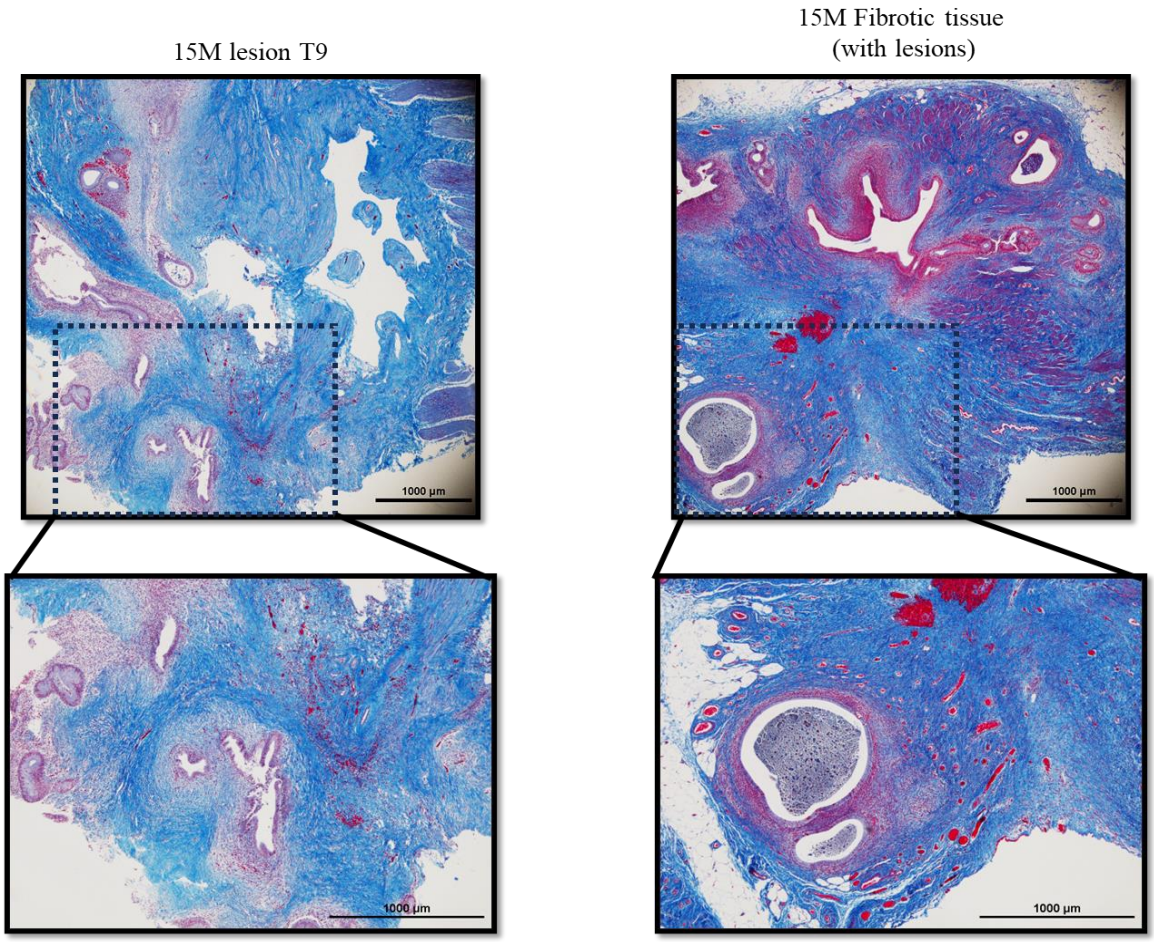

Figure 3.12: 15-month lesions with fibrotic tissue from the baboon model. We can observe the high deposition of collagen at 15 months and the intensity of fibrosis. n=5 per group and Scale bar: 1000µm

These lesions allowed us not only to confirm the presence of fibrosis in human endometriotic lesions, but they also provided images of different structures of the human lesions. The bottom lesion in figure 3.13 shows a cystic lesion compared with the other 2 lesions. This emphasizes the heterogeneity of the lesions and difficulty associated with their characterization in women. All the lesions showed deposition of collagen in the stromal area.

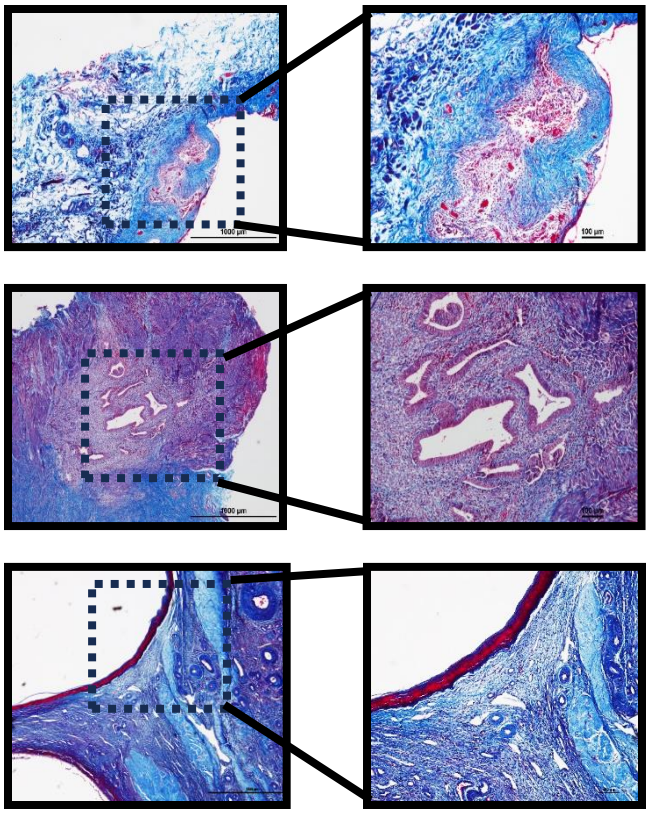

Figure 3.13: Masson trichrome staining of endometriotic lesions from women with endometriosis. We can observe the heterogeneity among the human lesions, n=3 and Scale bar: 1000µm

3.4 Discussion

Our study showed that miR-21 was upregulated in the stroma of endometriotic lesions from the mouse model of endometriosis, baboon, and women with endometriosis. In addition to this, we observed an association between miR-21 and fibrosis. Previous studies have shown a clear relationship between miR-21 and fibrosis in different diseases such as cardiac fibrosis[275, 276], scleroderma fibrosis[286], lung fibrosis[277] amongst others. This study is the first one to show a correlation between miR-21 and the development of fibrosis during the progression of endometriosis.

Fibrosis is one of the established hallmarks of endometriosis[114]. The development of fibrosis in endometriotic lesions is per se a complex phenomenon with

underlying mechanisms that still remain to be understood[3]. Fibrosis is present in different types of endometriosis, such as peritoneal fibrosis, ovarian fibrosis, deep infiltrating endometriosis (DIE) or adhering tissue. All these different manifestations of fibrosis could lead to pain, anatomical distortion, or infertility[287].

miR-21 is a very well established pro-fibrotic miRNA[288]. Studies have demonstrated that elevated expression of miR-21 may play an important role in the development of fibrosis[289]. Previous studies have demonstrated the importance of miRNAs during the progression of endometriosis[290]. The small-RNA seq analysis of baboon lesions performed in this study showed not only a group of miRNAS that are dysregulated during endometriosis, but also, the specific ones associated with fibrosis. One of the dysregulated miRNAs found in that analysis was miR-21. This outcome may indicate that the upregulation of miR-21 that we observed in the different models of endometriosis, may relate to the establishment of fibrosis in patients with endometriosis.

To study the relevance of miR-21 throughout the development of fibrosis during the progression of endometriosis, we utilized the Pgr $^{cre/+}$ Rosa 26$^{mT/mG}$ mouse model of endometriosis which develops endometriotic lesions similar to the ones observed in humans[129]. Utilizing this mouse model, we observed that the number of lesions that developed through the progression of endometriosis increases as the disease advances. In addition to this, we also utilized this model to quantify the levels of miR-21 expression in the lesions compared with matched uteri and confirmed that it was significantly upregulated after one month of induction of endometriosis. We observed that after three months of induction there are no differences. This could be due to the type of lesions selected for RT-qPCR. In the mouse model of endometriosis, during the development of

the disease, as in women, there are different types of lesions that are evident at the time of collection. As the disease advances, the lesions become more cystic. These types of lesions contain less stroma compared to lesions that are collected at earlier time points. At three months following the induction of endometriosis, we observed several lesions that were cystic. The non-cystic lesions collected at three months were primarily used for the *in situ* analysis since they have good histology. Figure 3.5 shows mouse lesions at three months with strong expression of miR-21. Having more cystic lesions at three months may be the reason why the miRNA levels of miR-21 are not significant at this time point.

When we compared the miR-21 expression results in the mouse and baboon models with the human lesions, we observed an increase in the levels of miR-21 expression although the increase was not statistically significant. Different studies have shown that miRNA expression pattern may differ depending on the endometriosis lesion type[291]. In this study, lesions collected from the mouse model and the baboon model were primarily peritoneal lesions. In the case of the women with endometriosis, the samples utilized for this analysis were derived from women with DIE. This finding suggests once again the importance of identifying the nature of the endometriotic lesions and the heterogeneity in the population of women with endometriosis. miR-21 is one of the miRNAs that can differ significantly across endometriotic lesion type[291].

Our in situ hybridization results showed a high expression of miR-21 in the stromal compartment of the mouse and baboon lesions. The location of miR-21 matched with the deposition of collagen based on the Masson trichrome staining indicating an association with fibrosis. One of our parallel studies also confirmed that this deposition of collagen

around mouse endometriotic lesions using gadolinium-based collagen I targeting probe[292]. Other studies have detected, using *in situ* hybridization, an upregulation of miR-21 expression predominantly in the stromal cell compartment, mainly in tumor-associated fibroblasts[293] [294]. In addition to this, other groups have suggested that miR-21 can influence fibrogenic processes in adenocarcinoma[295] and colorectal cancer[296]. These studies match our findings related to the role of miR-21 in fibrogenesis and support the hypothesis that miR-21 plays a role in the development of fibrosis in endometriosis.

Another component that was shown to be relevant in our studies was the presence of CTGF. The production of CTGF, a key marker in fibrotic disorders, such as endometriosis and intrauterine adhesions, is through STAT3-dependent Smad signaling[297]. This is important to emphasize because STAT3 can also stimulate the expression of miR-21[298]. Our data showed that CTGF is highly expressed in the stromal compartment in the mouse and the baboon models. This was verified with IHC of CTGF in the mouse model and the baboon and it was also confirmed with the RT-qPCR data from mouse, baboon, and women with endometriosis. This finding not only showed once again the advantages to use the mouse and the baboon model of endometriosis, it also validates our finding in the women with endometriosis.

The connective tissue growth factor also has been reported to enhance the mRNA expression of Collagen I and fibronectin in fibroblasts[299]. It is also known that TGF-β and CTGF are downstream mediators of TGF-β modulating fibroblast cell growth, extracellular matrix secretion and enhancing production of collagen[300]. TGF-β regulates the transcription of several genes involved in fibrosis, including CTGF [301].

This regulation is important because TGF-β can also regulate miR-21 in fibrotic diseases. Studies have suggested that CTGF acts as a mediator of TGF-β-induced fibrotic pathways via miR-21 regulation[272]. This suggests that there might be a potential connection between miR-21 and CTGF during the development of fibrosis in endometriosis.

In conclusion, our study showed that miR-21 is involved in the process of fibrosis during the development of endometriosis. Secondly, the expression of miR-21 was increased in endometriotic lesions in the mouse, baboon and women compared with the eutopic endometrium. Thirdly, CTGF and Collagen deposition are increased in expression during the development of the disease. Fourthly, miR-21 is primarily a stromal miRNA. Lastly, the localization of miR-21 in the stroma of endometriotic lesions matches with the deposition of collagen suggesting that miR-21 is involved in the process of fibrosis during the progression of endometriosis. These findings highlight miR-21 as a potential diagnostic and prognostic marker and therapeutic target for fibrotic diseases such endometriosis.

After confirming the key role of miR-21 during the development of fibrosis in the context of endometriosis, in the next chapter I investigate the mechanisms that could potentially regulate miR-21. From results obtained from the RNA-seq, we know that there is an important inflammatory component that is present during the disease. In the next chapter, we will show how some of those inflammatory components could be relevant in the regulation of miR-21 and how miR-21 could contribute to the fibrogenesis pathway.

CHAPTER 4: INTERLEUKIN-6 (IL-6) AND SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 (STAT3), REGULATES THE EXPRESSION OF MICRORNA-21 DURING THE DEVELOPMENT OF ENDOMETRIOSIS

4.1 Introduction

Endometriosis is the most common cause of chronic pelvic pain and infertility, which affects 10% of women during their reproductive years[4, 67]. Endometriosis is an underdiagnosed disease that is associated with 8–10-year delay from the onset of symptoms to diagnosis. This disorder is an estrogen-dependent inflammatory disease characterized by the presence of endometrial epithelial and stromal cells at ectopic locations that result in bleeding, inflammation, fibrosis and adhesion formation, primarily in the pelvic area[4].

Menstrual fluid of women with endometriosis contains, among other factors, inflammatory cytokines that cause changes in the peritoneal cavity promoting endometrial cell adhesion into the peritoneum[302]. The peritoneal fluid in women with endometriosis contains abundant proinflammatory cytokines, particularly interleukin 6 (IL-6), which create a chronic inflammatory state[303]. Chronic inflammation often leads to tissue fibrosis that can trigger an excessive accumulation of extracellular matrix components, which could lead to the formation of a permanent fibrotic scar[304]. Activated B cells are known to produce IL-6 and high concentrations increase of IL-6 may directly promote tissue fibrosis[305, 306].

IL-6 is a soluble mediator with a pleiotropic effect on inflammation and immune response[307]. IL-6 is the leading member of a family of cytokines that use gp130 receptor subunit as a signal transducer in the receptor complex[308]. The IL-6 receptor (IL-6R)

system includes, IL-6 binding chain, IL-6R and gp130 which constitutes the signal-transducing chain. The activated IL-6R complex forms an hexameric structure including two molecules each of IL-6, IL-6R and gp130[309, 310]. The activation of gp130 triggers the activation of downstream signaling molecules, including signal transducer and activator of the transcription 3 (STAT3) [307-309]. STAT3 is located in the cytoplasm until it is activated by phosphorylation. After activation, it translocates into the nucleus regulating and binding to promoter regions for target gene expression[190]. Previous studies have shown that STAT3 is significantly involved in inflammatory responses. It can be activated by different cytokines including IL-6, as we mentioned previously.

STAT3 is an important key transducer and regulator of genes that play an important role in processes such as cell development, proliferation, survival, and inflammatory processes[311]. It is not clear the role of STAT3 during the development of endometriosis, but studies have shown high levels of phosphorylated STAT3 (pSTAT3) in the endometrium of women with endometriosis compared with controls[190]. Different studies in baboons and mice have shown that the phosphorylation of STA3 can be increased by IL-6[190, 298, 312, 313].

Abnormal miRNA expression has been also associated with numerous human diseases including gynecological disorders[314]. miRNAs have been suggested to play an important role in the pathogenesis of endometriosis. Studies have shown that the presence of endometriotic lesions can alter miRNA expression in both the eutopic and ectopic endometrium of baboons induced with endometriosis. Some miRNAs were also reported to be altered in women with the disease[147]. miRNAs are endogenous noncoding small RNA molecules with important functions in the regulation of gene

expression in physiological and pathological processes[140]. One miRNA that has been the subject of considerable research in the reproductive system and in other organs is miR-21[315]. mIR-21 was one of the first mammalian miRNAs identified[316]. It is found on chromosome 17 (17q.23.1) in the 11th intron of the TMEM49 (transmembrane protein 49) gene, precursor of VMP1 (vacuole membrane protein 1[317].

Studies have shown that the induction of endometriosis using the baboon model, has resulted in the upregulation of different miRNAs, including miR-21[147]. Different studies (including chapter 3) have revealed that miR-21 is upregulated during fibrosis, a hallmark of endometriosis, and can regulate the fibrogenic process in a variety of organs and tissues through different pathways,[318] including the TGF-β pathway, promoting fibroblast proliferation[277, 319].

In other diseases, there is data that demonstrates that miR-21 gene transcription could be regulated by IL-6 via STAT3[312]. In this study, we hypothesized that the inflammatory environment driven by the upregulation of IL-6 during the development of endometriosis could cause the over expression of miR-21 via activation of STAT3 (Figure 4.1).

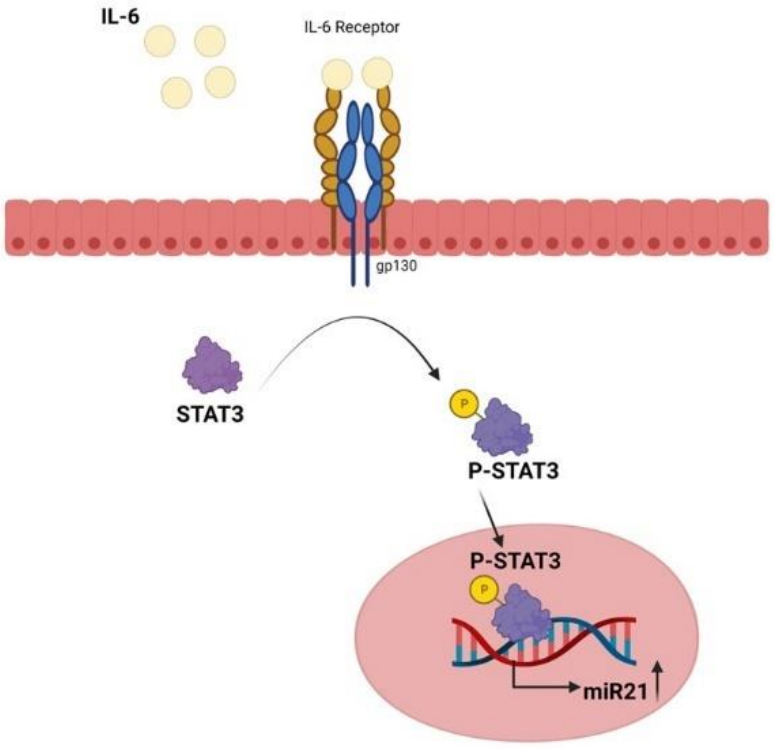

Figure 4.1: Working hypothesis of the inflammatory environment driven by the upregulation of IL-6 during the development of endometriosis causing the over expression of miR-21 via activation of STAT3.Created with BioRender.com

4.2 Materials and Methods

4.2.1 Cell culture and transfection

An immortalized human endometriotic stromal cell line (iEc-ESC)[320] was cultured with phenol red–free DMEM/F-12 (Gibco, USA) medium supplemented with 15% CDS-FBS (Gibco, USA), 100 U/mL of Pen/Strep (Gibco, USA), and 1 mM sodium pyruvate (Gibco, USA) at 37C under 5% CO2 and 95% air. Following optimization of parameters, Lipofectamine RNAiMAX (Invitrogen, USA) was used to transfect iEc-ESC with 25 pmol of miR-21 mimics (Ambion, USA) or with 25 pmol of nontargeting negative controls (Ambion, USA), and RNA and protein were isolated after 24 h. To check the expression of miR-21 qRT–PCR was performed. iEc-ESC cells were also plated at 3 ×

10^5 cells per well in 6-well plates. The following day, the cells were treated with recombinant human IL-6 (206-IL010, R&D Systems, Inc.) (50 ng/mL) in 2% charcoal-dextran treated fetal bovine serum DMEM/F-12 media. RNA and protein were isolated .

4.2.2 RNA extraction and qRT-PCR

Total RNA was isolated from frozen tissue or cultured cells using TRIzol reagent (Life Technologies). RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Quantitative PCR (qPCR) was then performed to measure gene expression levels with SYBR® Green PCR Master Mix (Applied Biosystems) using the ViiA7 qPCR System (Applied Biosystems). For measuring miRNAs transcript levels, 100 ng of total RNA was reverse transcribed using a TaqMan microRNA reverse transcription kit (Applied Biosystems, USA) as per the manufacturer's protocol. Following reverse transcription, miR-specific cDNA expression of mature miRNAs was analyzed using TaqMan miR Assays (Applied Biosystems, USA) and TaqMan universal PCR master mix (Applied Biosystems, USA). The miRNAs expression data were normalized using expression of endogenous U6 small nuclear RNA. For analysis of miRNAs target expression, total RNA (1000 ng) was reverse transcribed using a High-Capacity cDNA synthesis kit (Applied Biosystems, USA) according to the manufacturer's protocol. After the cDNA synthesis step, cDNA and qRT–PCR reaction was carried out for miR targets, Smad7, using TaqMan gene assays and TaqMan Universal PCR master mix (Applied Biosystems, USA). 18S ribosomal RNA (18S) and RPL17 were used as an internal control for normalizing qRT–PCR data. Primer sequences used for qPCR are listed in Supplementary table 3A. All quantitative real-time

PCR reactions were run for 40 cycles, and fold change was calculated using $2^{-\Delta\Delta Ct}$ method[284].

4.2.3 Western Blot Analysis

Cells were rinsed with ice-cold PBS on ice and the lysed with Pierce® RIPA lysis buffer (Thermo Fischer Scientific, Rockford, IL) supplemented with protease inhibitors and phosphatase inhibitors (Thermo Fischer Scientific). The protein concentration was measured using the Pierce® BCA protein assay kit (Thermo Fischer Scientific). Eight micrograms of protein per well were separated on 4% to 20% Tris-Glycine gels (Invitrogen) and transferred onto a polyvinylidene fluoride membranes (Millipore, Billerica, MA). The membranes were then incubated for 1 hour at room temperature in 5% BSA TBST buffer and then incubated overnight with primary antibodies against STAT3 (#124H6 CST), pSTAT3 (#9145s CST), SMAD7 (#MAB2029-SP R&D Systems), SMAD2(#5339 CST), pSMAD2(#18338 CST) and β-actin(#4967 CST) in blocking buffer overnight at 4°C. The next day, the membranes were incubated with the respective secondary antibodies labeled with horseradish peroxidase (Pierce®, Thermo Fischer Scientific) for 1 hour at room temperature. Immunocomplexes were visualized by enhanced chemiluminescence (GE Amersham, Piscataway, NJ). Protein bands were analyzed with Image J (National Institutes of Health). Protein levels of SMAD7 were normalized to β-actin as the internal control.

4.2.4 CUT&RUN Assay

The CUT&RUN assay was performed using a kit from Cell Signaling Technology (Cat#86652) and following the manufacturer's protocol[321]. Briefly, Concanavalin A–coated magnetic beads were treated with Bead activation buffer. Ectopic stromal cells

(~100,000 cells) were harvested at room temperature and resuspended in wash buffer containing Spermidine and protease inhibitor cocktail (PIC), and the cell pellet was washed twice. The concanavalin A bead suspension was added and mixed on a tube rotator for 5 min at room temp. Cell: bead conjugate suspensions were resuspended in Antibody Binding Buffer (Digitonin + Spermidine + PIC) containing 5µl of p-STAT3 antibody (CST cat#9145S) and control reaction with 5µl of Rabbit IgG (CST, cat# 66362S) and incubated in a tube nutator overnight at 4C. The following day, cell: bead suspensions were washed in Digitonin Buffer (+ Spermidine + PIC) three times, resuspended in Digitonin Buffer and 50µl of pAG-MNase pre-mix was added to each tube and gently mixed by pipetting up and down followed by placing the cells on a rotator at room temperature for 1 hour followed by two washes in Digitonin Buffer. MNase was activated by adding 3µl of cold Calcium Chloride to each reaction, and tubes were rotated at 4C for 30 minutes. Following this step, the beads were rotated at 37C for 10 min, followed by centrifugation at 16,000g for 2 min at 4C, and placed on a magnetic rack until the solution was clear. The enriched chromatin sample in the supernatant was transferred to a fresh 1.5ml microcentrifuge tube and processed for DNA purification using NucleoSpin Gel and PCR Clean-up kit from MACHEREY-NAGEL GmbH & Co. Germany (cat# 740609.50). The purified DNA was processed for the qPCR analysis using site-specific primer sets (Supplemental Table 4A), and data obtained were presented as fold enrichment compared to IgG controls.

4.2.5 Immunofluorescence Staining

Cells were grown on glass coverslips to 80–90% confluency. The coverslips were washed with PBS and fixed with 2% paraformaldehyde in PBS for 10 min at 37 °C. The

fixed cells were treated with blocking buffer (PBS/5% goat normal serum/0.3% of Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature. The cells were then incubated with the primary antibodies overnight at 4 °C and secondary antibodies for 1 h at room temperature. The primary antibody information is as follows: IL-6R antibody (#128008 abcam). The secondary antibody information is as follows: DyLight 594 goat anti-rabbit IgG antibody (DI-1594) and DyLight 488 goat anti-rabbit IgG antibody (DI-1488) were from Vector Laboratories (Burlingame, CA). PBS washed coverslips were then mounted onto microscope slides with a DAPI-impregnated mounting media (Vector Laboratories, Burlingame, CA) to enable nuclear visualization and images captured with a fluorescent microscope (Nikon Instruments Inc., Melville, NY) using software from NIS Elements, Inc. (Nikon, Melville, NY).

4.2.6 Induction of endometriosis in mice

Animals were maintained in a designated animal care facility according to the Michigan State University's Institutional Guidelines for the care and use of laboratory animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University. Eight-week-old mice were injected with estradiol (E2) (0.1µg/mouse) every 24 hours for 3 days, and then surgical induction of endometriosis was performed as previously described in chapter 3. Following the induction of endometriosis, 5 µg IL-6 (Recombinant Mouse IL-6 Protein 406-ML-005: R&D Systems) per injection or PBS (Veh) was injected IP twice per week for 2 weeks. After 2 weeks, mice were euthanized, the peritoneal cavity was opened, and endometriotic lesions were counted and removed under the dissection microscope.

Endometriotic lesions and uterine tissues were fixed with 4% (vol/vol) paraformaldehyde for histological analysis.

4.2.7 Statistical Analysis

Differences in miRNA and gene expression between control and endometriotic eutopic and ectopic endometrium were compared following normalization against U6 and RPS17, 18S, 36B, respectively. Statistical analyses were performed using Prism 8.3.4 (GraphPad Software Inc., USA). All data were expressed as mean ± SD. The student's 2-tailed t test was used for comparisons of 2 groups, and 1-way ANOVA with a least significant difference post hoc test was used for multiple comparisons. P value less than 0.05 was considered significant.

4.3 Results

4.3.1 IL-6 receptor is expressed in Ectopic stromal cells.

Cytokines can contribute to the pathophysiology of endometriosis in at least two ways, by enhancing the establishment and proliferation of ectopic endometrial implants and by influencing the secretion of cytokines by macrophages[322]. IL-6 is a multifunctional cytokine that stimulates cell proliferation and is involved in the formation of adhesions. It is known that IL-6 is elevated in the peritoneal fluid, endometriotic lesions, and serum from women with endometriosis[98, 323]. IL-6 exerts multiple bioactivities through its receptor (IL-6R). Membrane-binding receptor (mIL-6R) and soluble receptor (sIL6R) are 2 forms of IL-6R. The biological activity of IL-6 is mainly mediated through binding with the corresponding mIL-6R[98]. To confirm the presence of IL-6 receptor in ectopic stromal cells and conforming to their normal morphology (Figure 4.2A), they were

stained for IL-6R by immunofluorescence staining. Figure 4.2B shows that ectopic stromal cells were positive for IL-6 receptor.

4.3.2 IL-6 upregulates miR-21 via p-STAT3 in ectopic stromal cells

A variety of cytokines including interleukin-6 (IL-6), can activate the signal transducer and activator of transcription (STAT) family of transcription factors, specifically STAT3[324, 325]. STAT3 is localized in the cytoplasm until activated by phosphorylation. On activation, STAT3 are phosphorylated by Janus kinase (JAK) and then form homo- or heterodimers that translocates to the cell nucleus, where they act as transcription activators[313].

To confirm this in our cell lines, we first initiated a dose response with 25 ng/mL and 50ng/mL of IL-6 (Figure 4.3A). Both of the concentrations resulted in a positive response, but 50ng/mL had the higher response. A time course experiment (12, 24 and 28 hours) with 50ng/mL was also performed. Following IL-6 stimulation at 50ng/mL, miR-21 was initially increased (Figure 4.3B) at 12 hours and one of its targets, Smad 7, was significantly decreased. To test these results *in silico*, STAT3 binding sites on miR-21 promoter regions on chromosome 17 (Figure 4.4A) were identified using the genome browser Ensembl (Figure 4.4B) and the transcription factor binding profile database JASPAR CORE 2022 (Figure 4.5A). To confirm this in silico observation, ectopic stromal cells (~100.000 cells) were processed for CUT&RUN assay by treating the cells with IL-6 and using p-STAT3 antibody. CUT&RUN assay is a relatively new technique to examine gene regulation including transcription factor binding.

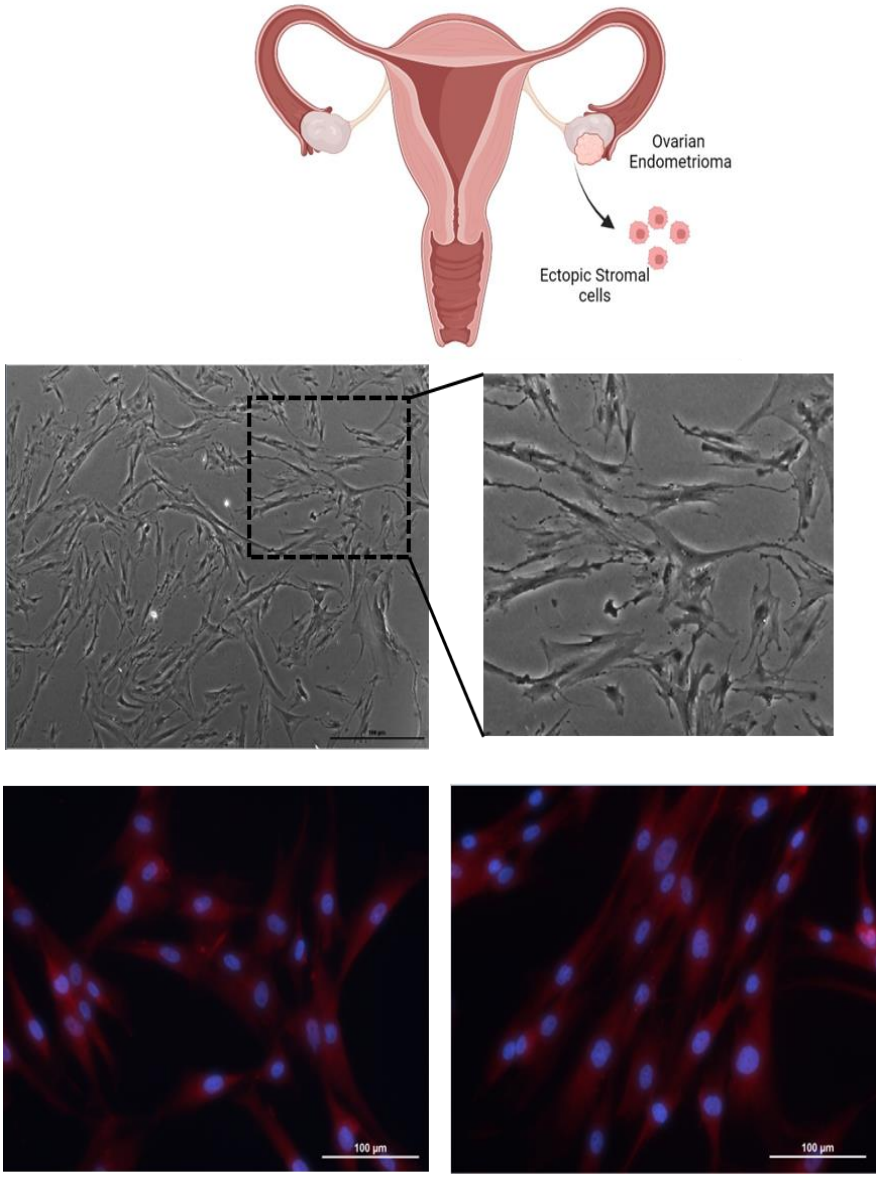

Figure 4.2: Representative images of primary human endometriotic stromal cells. (A) Brightfield image demonstrates the fibroblast-like morphology. Scale bar 500µm. (B) Immunofluorescence staining shows endometriotic stromal cells are positive for IL-6 receptor. Scale bar: 100µm

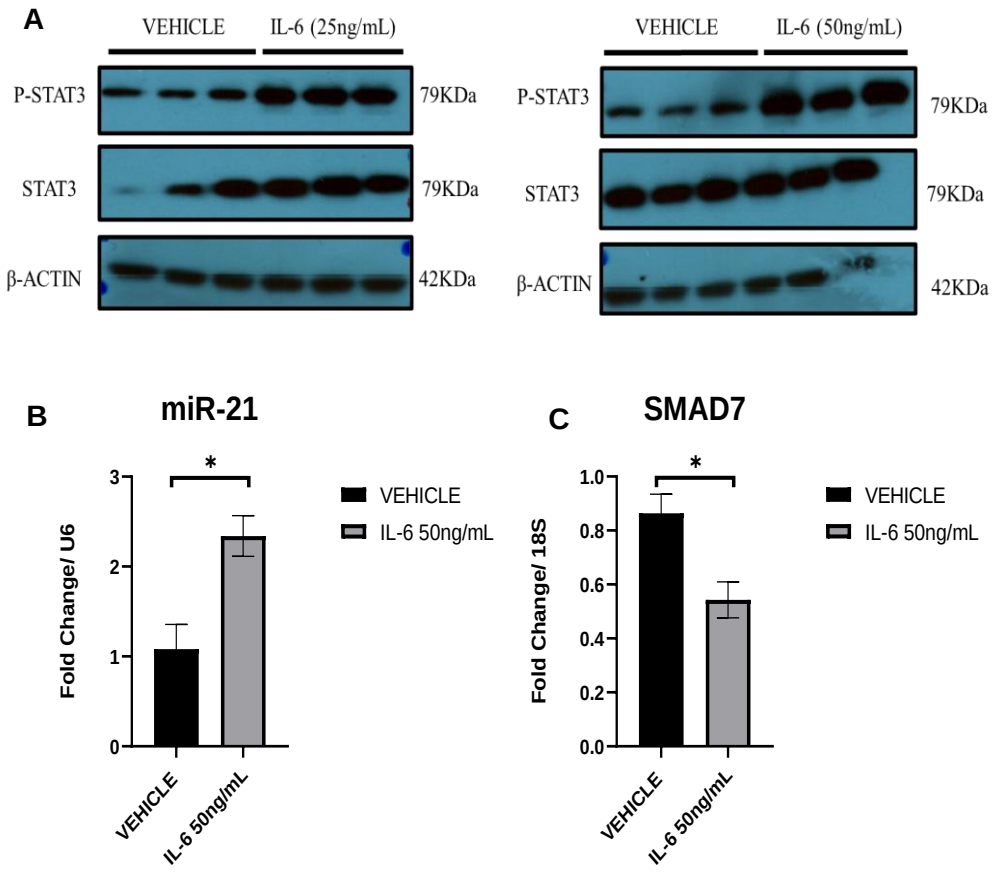

Figure 4.3: Regulation of miR-21 expression via P-STAT3. (A) Western Blot analysis of P-STAT3 and STAT3 in ectopic stromal cells in the presence of IL-6 recombinant at 25 ng/mL and 50 ng/mL. (B) qRT-PCR analysis of the miR-21 expression ($p<0.05$). (C) qRT-PCR analysis of the miR-21 target, SMAD7 expression($p<0.05$).

Ectopic stromal cells were treated with IL-6 (50ng/mL) for 12 hours and were harvested for the CUT&RUN assay. This in vitro experiment confirmed the regulation of miR-21 by IL-6 via p-STAT3 in the ectopic stromal cells (Figure 4.5B). From the motif map analysis, we found predicted STAT3 binding sites on the human miR-21 promoter region (Figure 4.5C, D). The CUT&RUN assay confirmed that STAT3 binds the transcription factor sites and revealed a 30-fold enrichment for p-STAT3 compared with the IgG control antibody (Figure 4.5E).

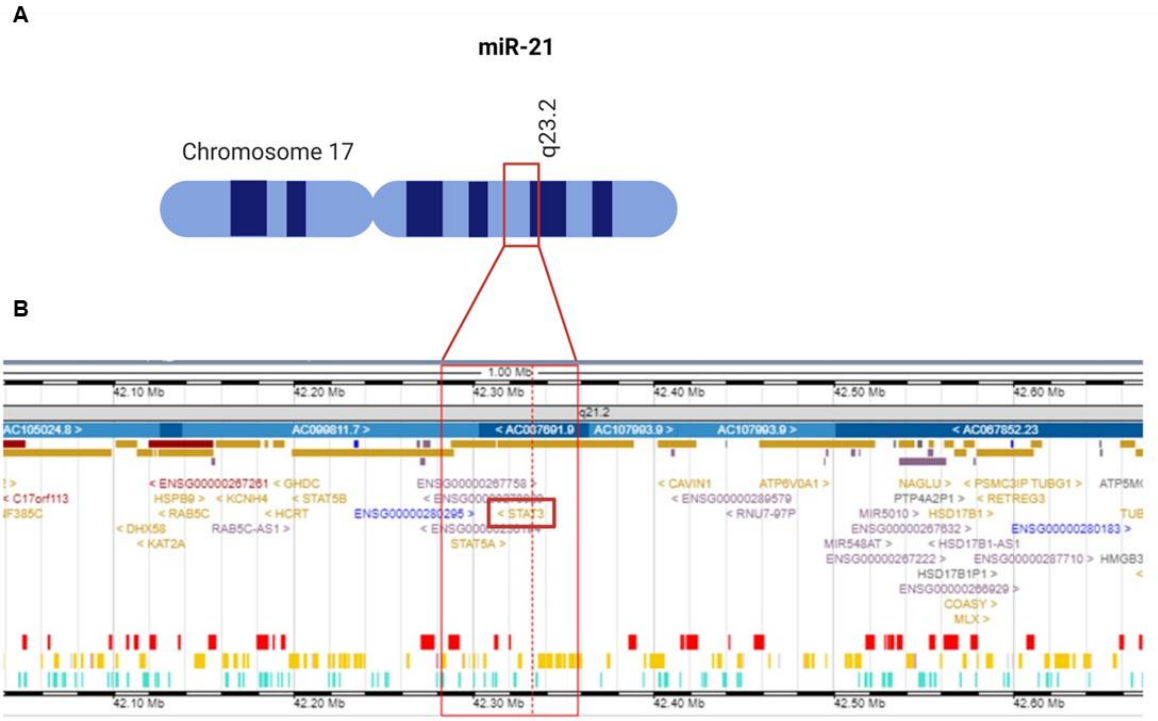

Figure 4.4: Genomic location and Epigenetic Landscape of miR-21. (A)miR-21 is located on chromosome 17 (17q.23.1) in the 10th intron of the TEM49 gene precursor of the VMP1 protein. (B)The putative miRNA promoter region containing STAT3 binding sites.

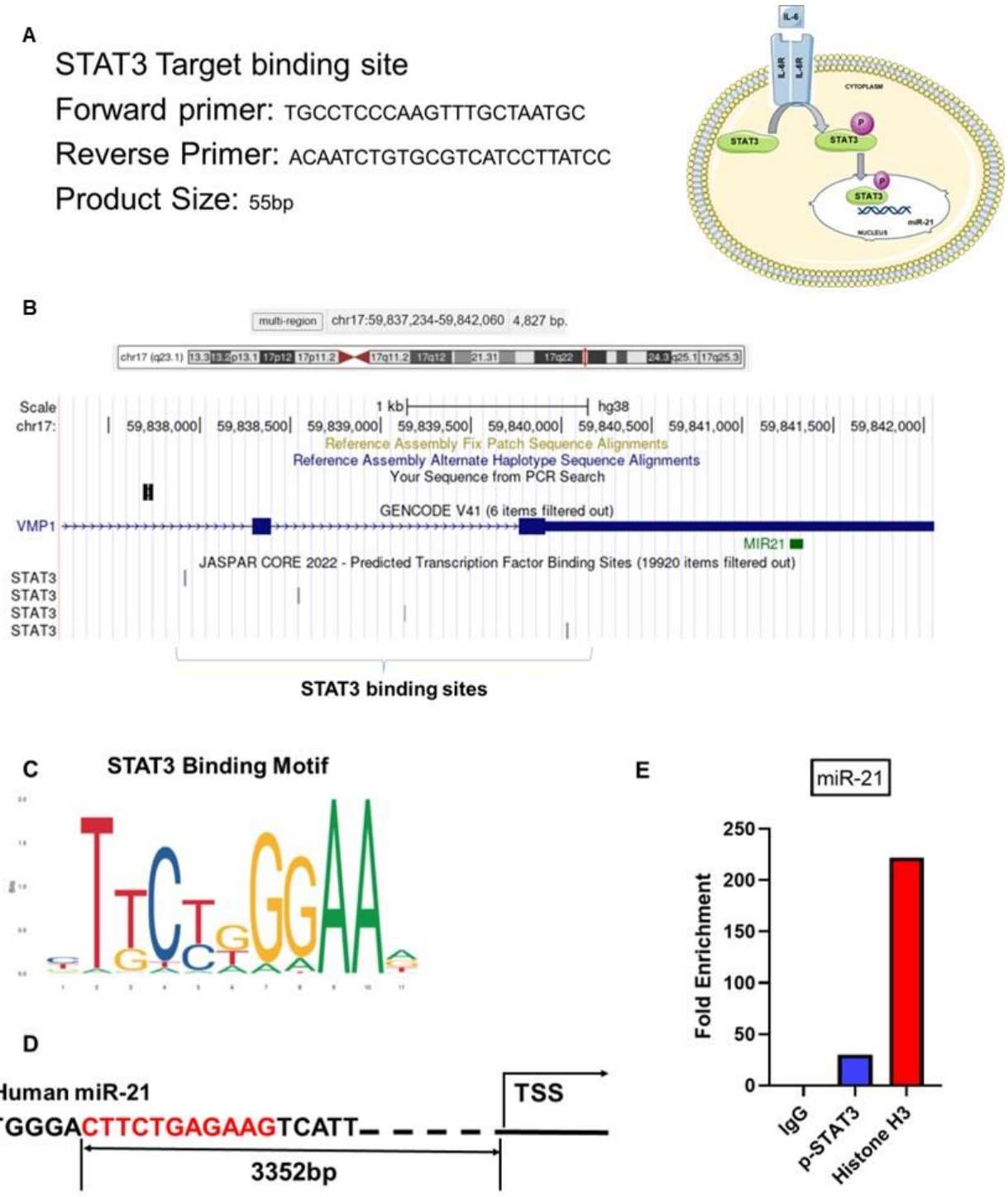

Figure 4.5: Predicted Transcription Factor binding sites for STAT3 (A) Results from the transcription factor binding profile database JASPAR CORE 2022 (B) Primer used for the binding site and overall summary of in vitro data obtained from the present study that suggests that the expression of miR-21 is increased in ectopic stromal cells via p-STAT3 binding**(**C, D) Predicted STAT3 binding site of human miR-21 promoter(E)Binding efficiency of STAT3 on the human miR-21 promoter enhanced by IL-6 stimulation

4.3.3 Upregulation of miR-21 in ectopic stromal cells.

Our in vitro data from the treatment of ectopic stromal cell with IL-6 demonstrated an increase of miR-21 expression and a decrease of Smad7 at mRNA level. After that, we performed a transfection with the miR-21 mimic or nontargeting negative controls to verify the previous results. The transfection with miR-21 mimic resulted in an increased detection of miR-21 (Figure 4.6A) and in a decrease of SMAD7 at mRNA level (Figure 4.6B). At mRNA level, these results matched with the results obtained with the IL-6 treatment.

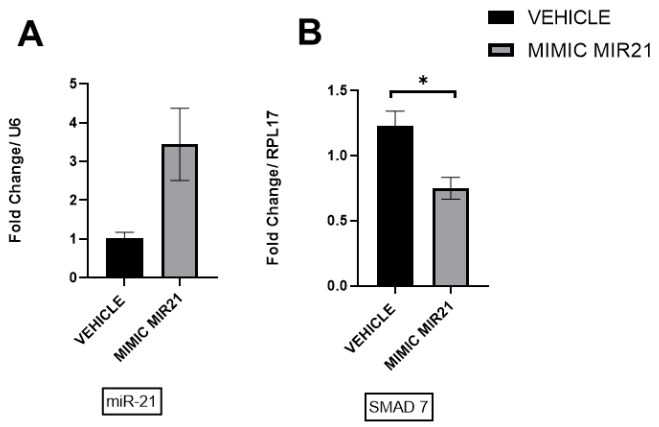

Figure 4.6: Overexpression of miR-21 in ectopic stromal cells. (A) Upregulation of miR-21 (B) Downregulation of Smad7 at mRNA level.

These results suggested that miR-21 downregulated SMAD7 in ectopic cells but not at the translational level (Figure 4.7A). Based on these results, we decided to analyze the downstream target of SMAD7, P-SMAD2, to verify this data. We observed that at transcriptional level SMAD7 and P-SMAD2 appeared to be downregulated (Figure 4.7B).

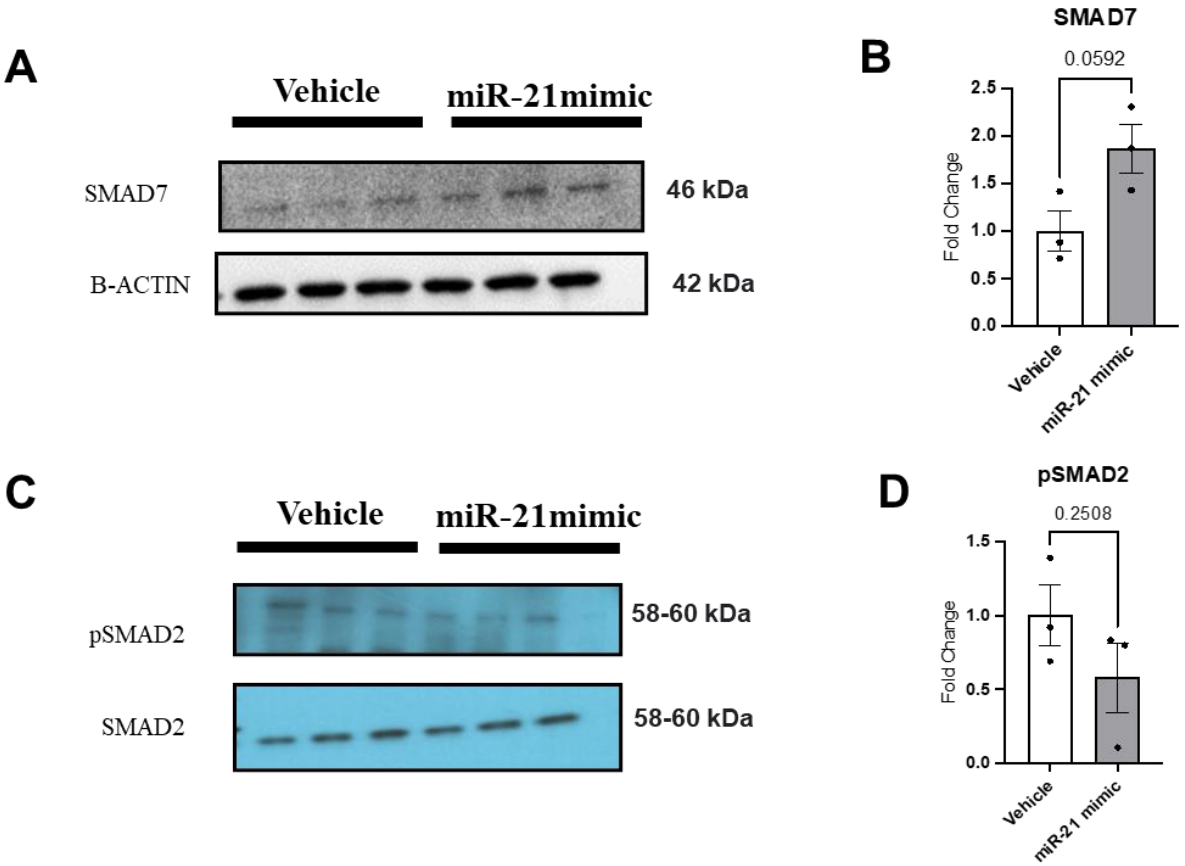

Figure 4.7: Overexpression of miR-21 in ectopic stromal cells.(A) Expression levels of Smad7 at a protein level. (B) Densiometric analysis of SMAD7 showing no differences in expression. n=3(C)Expression levels of p-SMAD2 and SMAD2. (D) Densiometric analysis of p-SMAD2 showing no differences. n=3.

4.3.4 IL-6 upregulates miR-21 in the mouse model

We have demonstrated that IL-6 can upregulate miR-21 in vitro. We used the mouse model of endometriosis to further confirm these results. Following induction of endometriosis in the mouse model (Figure 4.8A), we treated the animals with IL-6 (5µg/injection) or PBS into the peritoneal cavity for 15 days (Figure 4.8B). We observed that the mice treated with IL-6 had a significant increase of the number of lesions compared with the PBS controls (Figure 4.9A,B). The weight of the lesions pooled together was also higher than the PBS group. In addition to this, the expression levels of miR-21 in the animals injected with IL-6 was significantly higher than the animals injected

with PBS (Figure 4.10A). The lesions were easily visualized in green under the fluorescence microscope (Figure 4.10B).

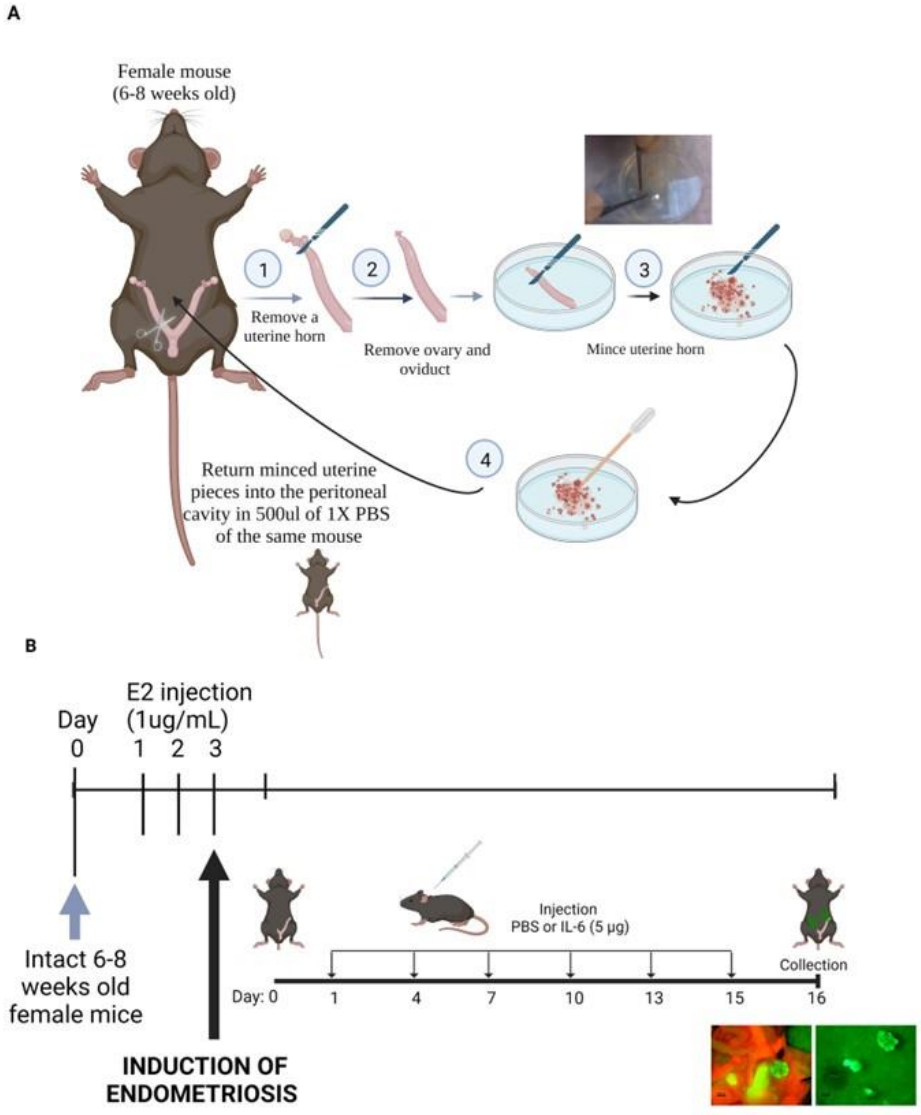

Figure 4.8: Treatment with IL-6 in the mouse model of endometriosis. (A)Schematic diagram of induction of endometriosis (B) Experimental design of the mouse model of endometriosis and the days of injection of IL-6 or PBS after endometriosis induction. Created with BioRender.com.

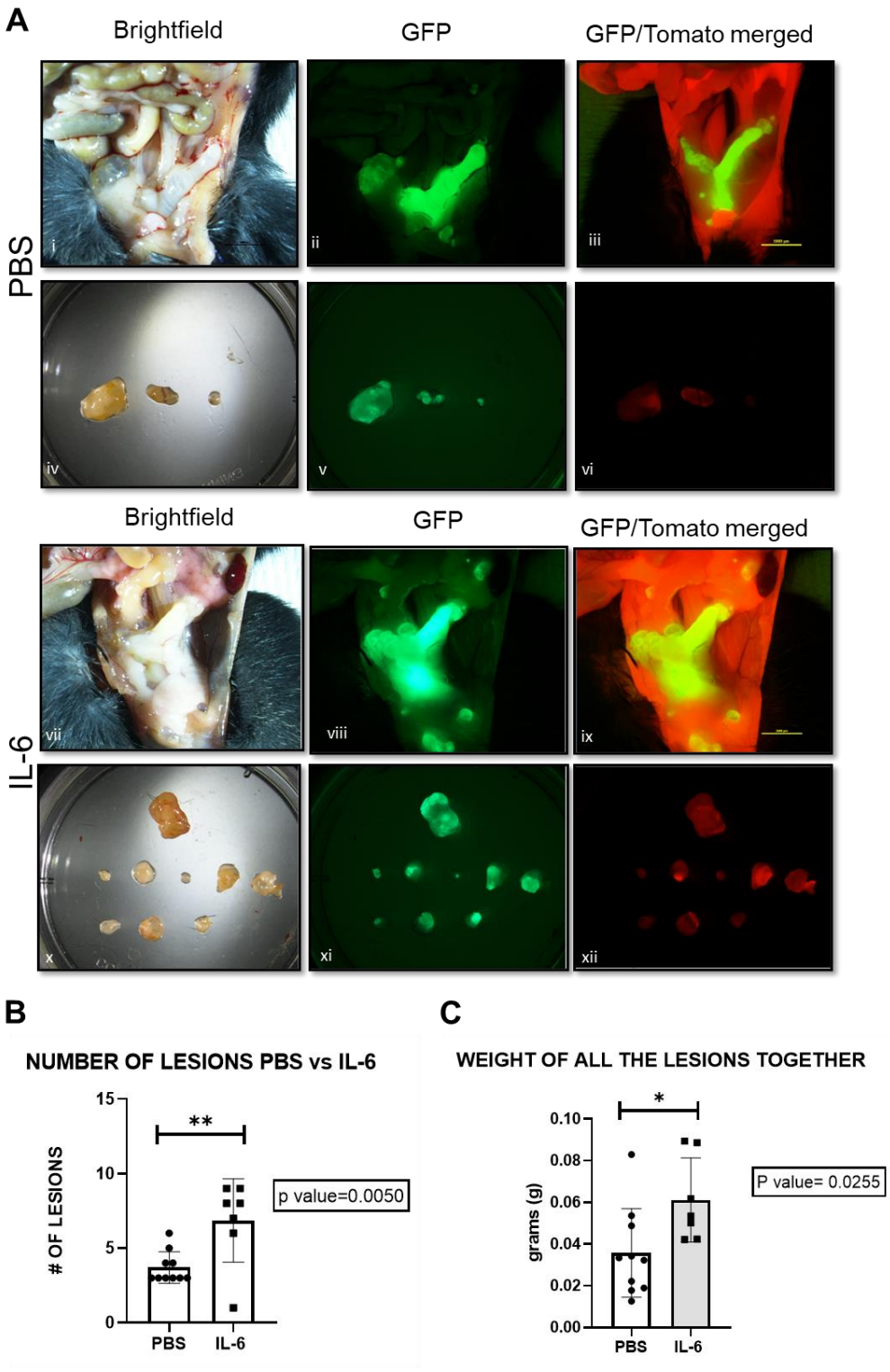

Figure 4.9: Mouse lesions and uterine cavity from PBS and IL-6 treatment (A) Representative picture in brightfield (I, iv, vii, x) GFP (ii, v, vii, xi) and GFP/Tomato merged

Figure 4.9 (cont'd)
(iii, vi, ix, xii). (B) Number of lesions in mice injected with PBS or IL-6. (C) Weight of all the lesions pooled together in animals injected with PBS or IL-6.

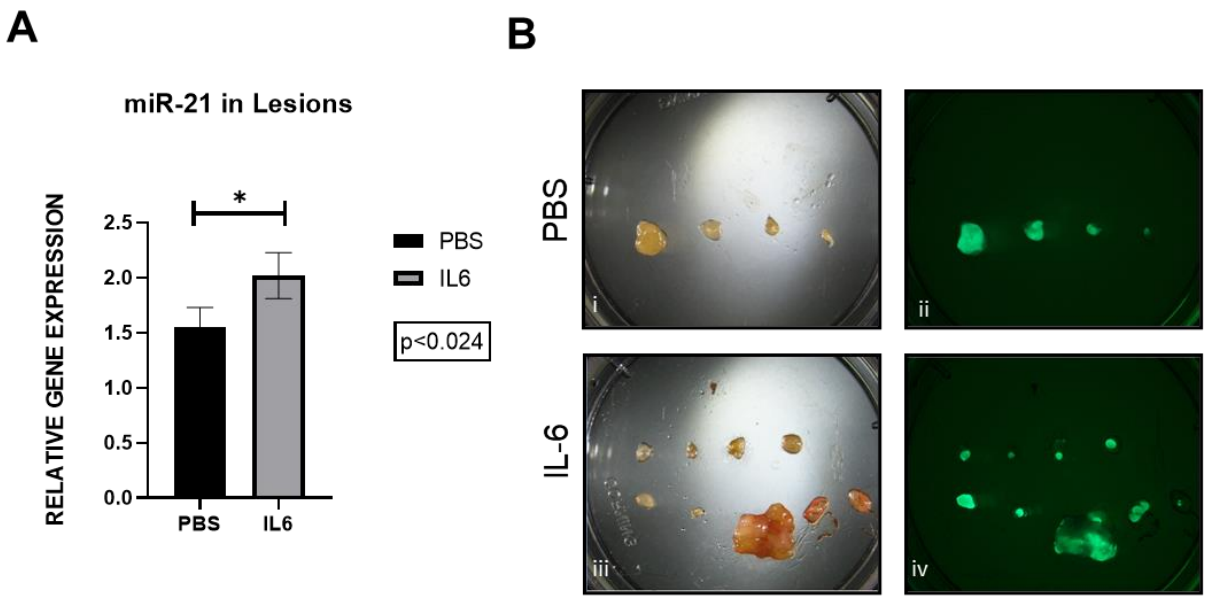

Figure 4.10: Comparison between mice injected with PBS and IL-6. (A) Expression of miR-21 in PBS or IL-6 treated mice. (B) Representative pictures of lesions from PBS and IL-6 mice in brightfield (i, iii) and under fluorescence GFP(ii, iv).

4.4 Discussion

In this present study, we investigated whether the expression of miR-21, via STAT3, is affected by the presence of IL-6, simulating the inflammatory environment that occurs during the development of endometriosis. Several studies have shown elevated levels of IL-6 in peritoneal fluid of patients with endometriosis[326, 327].

Macrophages are the predominant cells secreting IL-6 in peritoneal fluid[328] but B cells are also implicated in its secretion [304].The local pelvic inflammatory process, accompanied by altered function of immune-related cells and changes in cytokine content in the peritoneal cavity, have been shown to be related to the development of endometriosis[329]. As stated previously, endometriosis is an estrogen-dependent

disease with inflammatory components that play an important role in the pathogenesis of the disease[4]. There are a variety of cytokines including IL-6, that can activate the signal transducer and activator of transcription (STAT) family of transcription factors, such as STAT3[325].

In this study, we saw that this occurs in the ectopic stromal cells. The transcription factor, STAT3 is located in the cytoplasm until it is activated by phosphorylation. Once its activation occurs, it translocates to the nucleus and binds to promoter regions for target gene expression[330]. This results in the expression of miR-21. In our in vitro experiments, we confirmed this by treating the cells with IL-6. We observed that STAT3 was activated showing a higher expression of p-STAT3 in the cells treated with IL-6. At transcriptional level, we also confirmed the expression levels of miR-21 were upregulated. In addition, our CUT&RUN experiments confirmed this regulation as well. In these studies, we confirmed the regulation of miR-21 expression by IL-6 via p-STAT3 in the ectopic stromal cells.

In the present study, we also investigated the effect of IL-6 in vivo, using the mouse model of endometriosis. In this case, not only was miR-21 increased in lesions following 2 weeks of IL-6 treatment, but this was also accompanied with an increase in the number of lesions and an increase of the total weight of lesions. The fluorescence reporter genes that the mouse model utilized in this study allowed us to visualize in vivo endometrial lesions like those found in humans. Both *in vitro* and *in vivo* studies showed similar responses confirming the implication of IL-6 regulation in miR-21.

SMAD7 is one of the targets of miR-21, which contributes to fibrosis by targeting Smad7 regulation[331]. The next step was to demonstrate that miR-21 targeted the key

inhibitory protein SMAD7 in the ectopic stromal cells and promote the fibrotic process which miR-21 regulates via SMAD2. miR-21 usually downregulates SMAD7, promoting EMT and ECM deposition[332]. Different studies have performed dual luciferase reporter gene assays confirming that miR-21 can bind directly to the 3' UTR of SMAD7[331, 333]. In our study, when cells were treated with IL-6, we observed a significant downregulation of SMAD7 at transcriptional level. In addition, we also performed an additional in vitro experiment by overexpressing miR-21 in these cells. We verified that the effect was similar. SMAD7 showed a significant decrease in expression following the overexpression of miR-21 at transcriptional level. However, at protein level we did not observe differences in SMAD7 or p-SMAD2. These results could not directly confirm how SMAD7 contributes to the regulation of the TGF-β pathway.

Another possibility could be that different factors might be intervening in the regulation of SMAD7 at protein level independent of the effect that miR-21 has on SMAD7. There are studies that have shown that the presence of other factors, such as BMPs could upregulate SMADs such as SMAD7[332].

In vitro studies showed a significant regulation of miR-21 and how its target SMAD7 is downregulated at transcriptional level. Acknowledging the limitations of the antibodies utilized in these experiment and the difficulty to detect endogenous protein such as SMADS[334], more studies will be needed to understand the regulation of SMAD7 by miR-21 and to verify if that is the pathway by which miR-21 is promotes the development of fibrosis.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary

In this dissertation, I have focused on the two of the most relevant hallmarks of endometriosis, fibrosis, and inflammation. I analyzed the role that inflammation can play in the peritoneal cavity of women with endometriosis and more specifically, in regulatory miRNAs and its potential role in the development of fibrosis. Most of the focus here was placed on the role of the proinflammatory interleukin IL-6, due to the elevated levels in the peritoneal cavity of women with endometriosis[98, 190] and miR-21, a very well-known profibrotic miRNA and its potential role in the context of endometriosis[275-277].

In chapter 1, I navigated through the different postulated origins that could be contributing to the initiation of endometriosis and the possible hypothesis that have been discussed and questioned. I explained the pathophysiology of endometriosis by introducing the role that hormonal dysregulation plays in this disease. I highlighted the role of the inflammatory environment and the immune dysregulation during the development of endometriosis and the relevance of the fibrotic component during lesion development, and how all these factors together can contribute to infertility. In addition to this, I emphasized the importance of miRNAs in endometriosis and the current management and treatment of the disease. Lastly, I presented the different models used to study endometriosis, primarily the baboon and the mouse model and how they are contributing to expanding the knowledge in the field.

After introducing a broad landscape of endometriosis and emphasizing the importance of the animal models and more specifically the mouse, in chapter 2, I used the mouse model of endometriosis to understand transcriptomic changes that play a key role during

the early stages of lesion development. Here, I performed RNA-seq analysis in one-month lesions from the mouse model and identified pathways that are key for the development of fibrosis and for the inflammatory response are crucial at the initiation of the disease. These findings matched with other studies that have observed similar outcomes[210, 335, 336]. An unexpected finding in the transcriptomic analysis was the significant increase in B cells. B cells may be involved in the systemic and local production of cytokines in endometriosis, such as IL-6[304], and could potentially contribute to the inflammatory environment[88]. Despite B cell dysregulation which may be implicated in endometriosis, most of the previous studies have focused on peripheral B cells instead of endometrial B cells[239, 337]. This implies that to better understand the role of the immune system in the pathogenesis of endometriosis, the role of B cells should not be ignored since their role in the development of the disease is not well understood[338].

Chapter 3 was focused on the regulation of the fibrotic response that was observed in the RNA-seq data. Fibrosis is a common and intractable condition associated with various pathologies. It is characterized by accumulation of an excessive amount of extracellular matrix molecules that primarily include various types of collagen [339]. Here, I identified miR-21 as a potential contributor to the development of fibrosis within endometriotic lesions. Previous studies in our laboratory showed the relevance of miRNAs in this disease and how its dysregulation can play a key role in endometriosis pathology[53, 147]. In those studies, some miRNAs, including miR-21 were reported to be altered in women with endometriosis. Knowing the profibrotic nature of this miRNA, the *in situ* hybridization results verified that miR-21 was upregulated in baboon endometriotic

lesions and in the mouse lesions and this was correlated with fibrosis in the context of endometriosis.

With the aim to investigate these observations further and identify the components that could upregulate miR-21, the studies in chapter 4 were focused on the upstream pathways of miR-21 regulation. One of the pathways that I identified in the RNA-seq analysis was the presence of B cells. As mentioned previously, these cells can contribute to the secretion of IL-6. In this chapter, after performing CUT&RUN, I showed that IL-6 can upregulated miR-21 via STAT3. This in vitro experiment verified what other groups showed in other diseases[312, 340]. I showed that IL-6 signaling is important for the upregulation of miR-21 and that effect is dependent on STAT3.

5.2 Conclusions

In this dissertation, I hypothesized that the inflammatory environment in the peritoneal cavity of women with endometriosis promoted by IL-6, could upregulate miR-21 via STAT3, leading to an increase of fibrosis in endometriosis (Figure 5.1). The goals of my studies were: to determine the mechanisms by which the inflammatory environment driven by IL-6 in endometriosis can upregulated miR-21, to determine how miR-21 can modulate the TGF-β pathway by the inhibition of Smad7 to enhance fibrosis in the context of endometriosis and lastly, to understand the interplay between the TGF-β pathway-miR21 and CTGF and how they contribute towards endometriosis associated fibrosis.

The studies in this dissertation have contributed to a continuously growing body of knowledge of possible factors that regulate fibrosis in the context of endometriosis. We are the first group to show a correlation between miR-21 and its implication in the development of fibrosis in the context of endometriosis. Although we were not able to

clearly show the mechanism by which miR-21 promotes fibrosis (Figure 5.1B), we acknowledge that it is possible that the cell line utilized had some limitations. We must consider that the ectopic stromal cell utilized in this study has an origin in an endometrioma which may indicate that there might be other cell lines more suitable to study the fibrotic pathways.

While these studies also discovered new connections between IL-6 and miR-21, more work is needed to fully understand how the inflammatory environment can modulate the action of miRNAs. In addition, our findings align with other recent studies that showed that a persistent activation of STAT3 via IL-6 signaling is involved in fibrosis in endometriosis[341].

The RNA-seq revealed that B cells could potentially be a major provider of IL-6 in the peritoneal cavity promoting that initial inflammatory environment that would potentially upregulate miR-21. Although there are some studies that have demonstrated an abnormal production of autoantibodies in endometriosis, there is no consensus about the concentration of B cells and their role in this disease[239]. Research suggests that endometriosis has an autoimmune etiology, causing changes in humoral and cellular immunity leading to inflammatory reactions and proliferation of endometriotic cells[342, 343]. Common characteristics between endometriosis and autoimmune disease, such as tissue damage, activation of B cells, abnormalities of B and T cells, changes in apoptosis, multiorgan involvement, possible environmental factors and possible genetic basis could complete the classification criteria for endometriosis to be an immune disease[342, 344]. Findings from our studies would support this. We have observed that the genes differentially expressed in the one-month lesions are involved in organismal injury and

abnormalities in connective tissue disorders, which are associated with tissue damage and on inflammatory response.

The initial events that cause inflammation and fibrosis in endometriosis remain unknown. This dissertation has contributed to a better understanding to those components but does not conclusively answer the question. However, our data provided some key components that until now were unknown. We must acknowledge that every woman is different, the severity of the disease varies and the type of lesions that can be found in patients with endometriosis are completely heterogeneous. In this dissertation, we emphasized that miRNAs could play an important role in fibrosis, especially miR-21.

Taken together, the studies reported in this dissertation provided the following main findings: First, Inflammation and fibrosis are present at a very early stage of endometriosis. Secondly, B cells likely play an important role in the etiology and/or progression of endometriosis and should be further investigated. Third, the inflammatory environment lead by IL-6 can upregulate miR-21. Four, there is a correlation between miR-21 and fibrosis within endometriotic lesions. Five, there is clear advancement of fibrosis overtime. Six, the mechanisms by which miR-21 can lead fibrosis are not clear. Lastly, there might be other components implicated in the process that may play an important role in the development of fibrosis.

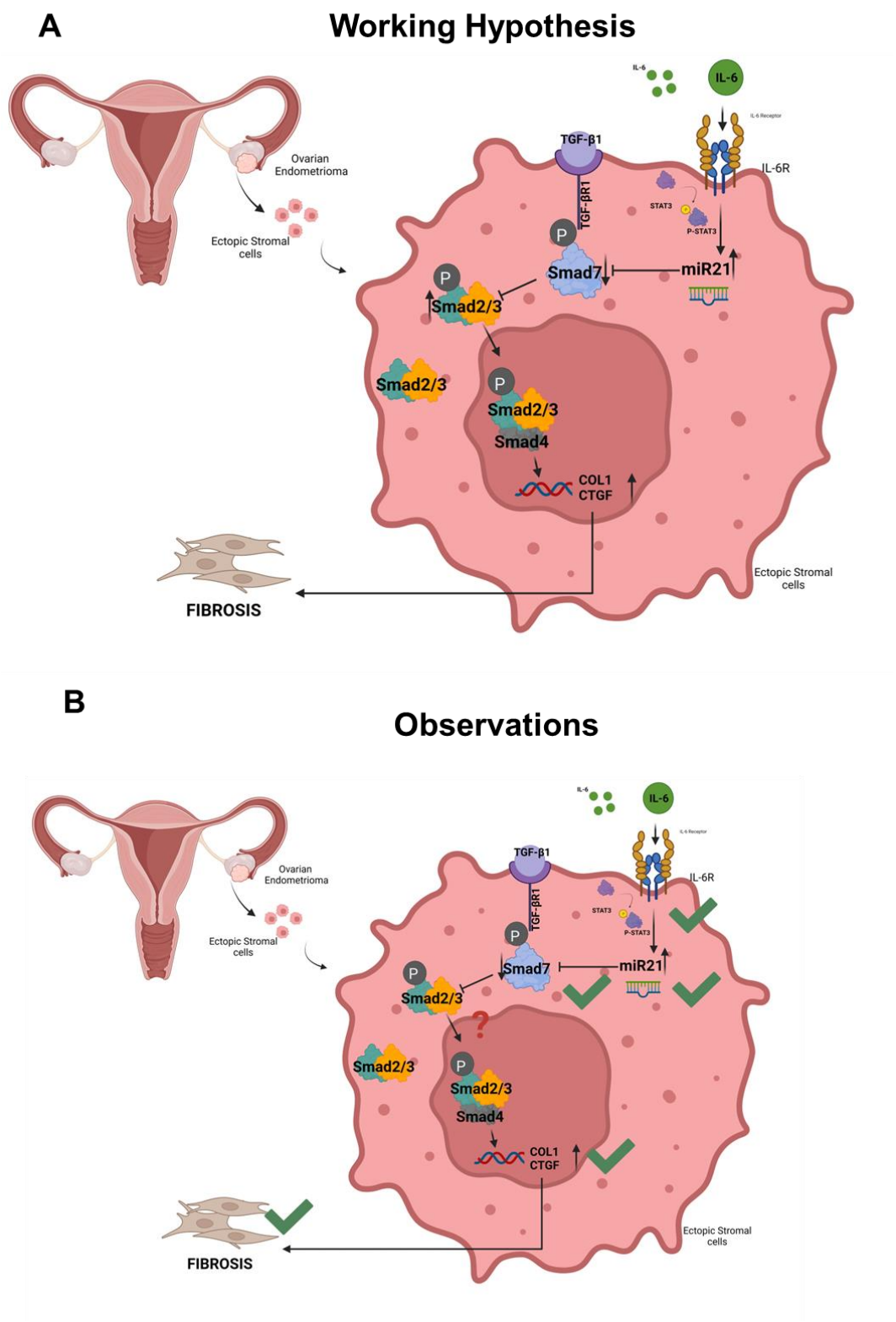

Figure 5.1: Summary figure. (A) Initial working hypothesis(B) Observations. Created with Biorender.com.

5.3 Future directions

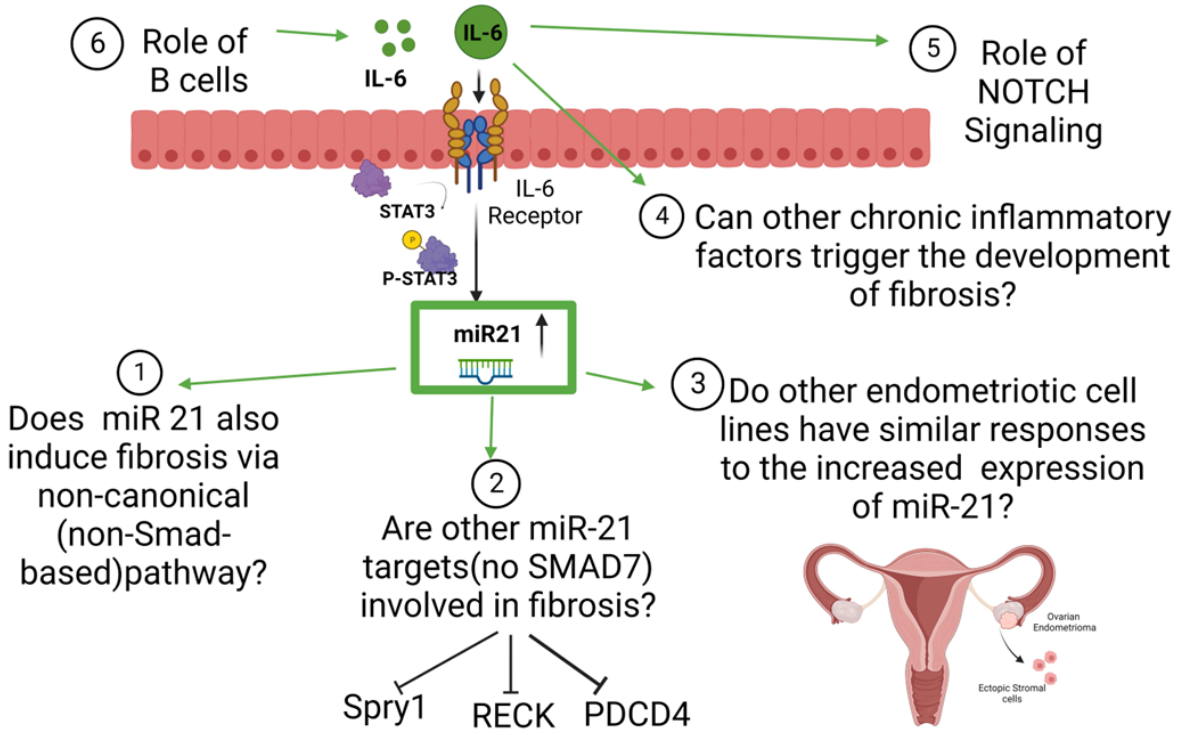

Figure 5.2: Proposed mechanisms for future directions. Created with Biorender.com.

5.3.1 miR-21 as a contributor of fibrosis using a non-canonical (non-Smad based) pathway

The data obtained here have contributed to the knowledge of the role that miR-21 plays in fibrosis. However, it was not clear that miR-21 regulated the TGF-β signaling pathway in the ectopic stromal cells. Other studies have reported that other factors such as Bone morphogenic proteins (BMPs) can significantly upregulate SMAD7[332]. We should consider that there might be other molecules that may be affecting miR-21 or may be contributing to fibrosis through alternative pathways. In addition there are studies that have observed dysregulation of SMAD7 in hypoxic conditions[345] and it is very well,

known that hypoxia plays critical roles in promoting pathological processes to facilitate the development of endometriosis[346].

5.3.2 Other miR-21 targets involved in fibrosis.

The involvement of miR-21 in the regulation of gene expression and its association with various functions suggest that miR-21 may accelerate the progression of tissue fibrosis[289]. Upregulation of miR-21 can promote tissue fibrosis by inhibiting its various targets. There are several well-known miR-21 targets (in addition to SMAD7) that have been validated in tissue fibrotic diseases, such as Spry1 or PDCD4. Identifying additional targets of miR-21 and interrupting the reciprocal loop between these factors are essential for a complete understanding of miR-21 function. We also must consider the context of miR-21. Specific targets of miR-21 may be different in distinct tissues or cell types. For instance, one miR-21 target can act as a target in a specific cell type, but not be a target anymore in a different type of cell. In our studies, we not only were able to validate that SMAD7 was a target of miR-21, but we also observed that PDC4 responded to our miR-21 mimic experiment and acted as a target (Figure 5.3). In the context of ectopic stromal cells PDCD4 is also a target of miR-21. There are several studies reporting the implication of miR-21 in fibrosis using different targets depending on the disease[275, 347]. This opens another possibility by which miR-21 may be involved in fibrosis. Elucidating the miR-21 targets and their involvement in pro-fibrogenic signaling is crucial. It also may provide novel perspectives for the understanding and treatment of fibrotic diseases.

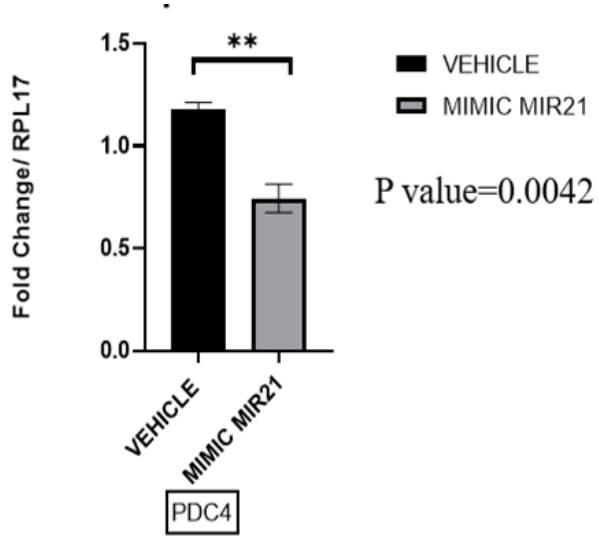

Figure 5.3: Downregulation of the miR-21 target, PDCD4 in ectopic stromal cells

5.3.3 Use of a different cell line

As mentioned before, targeting could depend on the context and the type of cells. In our case, we used ectopic stromal cells directly isolated from an endometrioma. One of the limitations of our study is that there is a possibility that since we were working with a cell line that came from a disease condition, it is possible that signaling pathways related with fibrosis might already be affected or altered in the ectopic stromal cells. Considering the same type of experiments and testing our hypothesis using a “no disease” endometriotic stromal cell could be a future possibility to consider.

5.3.4 IL-6 as a main contributor of fibrosis

Part of this dissertation has been focused on investigating the role of Il-6 in the upregulation of miR-21. IL-6 is described as a classic proinflammatory cytokine, however, it is also profibrotic, but the exact underlying mechanism is still undefined[339]. There are studies that have shown that IL-6 can modulate TGF-β expression in fibroblasts[348]. This opens the possibility that IL-6 could play an important role in fibrosis by itself or intervene in other pathways enhancing fibrosis. There are studies that support the

hypothesis that IL-6 could function as a regulator of normal dermal fibrotic repair and further suggest that IL-6 could play a role in the pathophysiology of dermal fibrotic diseases such as scleroderma or keloids[305].

5.3.5 Notch signaling as a contributor to fibrosis.

Studies from our laboratory have shown that *in vitro* and *in vivo* treatment with IL-6 can increase NOTCH1[99]. The NOTCH family of transmembrane receptors (NOTCH1-4) transduces extracellular signals and NOTCH signaling controls multiple cell fate decisions such as proliferation, survival, and immune modulation[349]. There are studies that have shown that ectopic endometrium from patients with endometriosis demonstrated hyperactivation of NOTCH signaling, which leads to fibrosis and inhibition of NOTCH cleavage can reduce fibrosis in ectopic endometrial stromal cells[350]. Some preliminary data from our laboratory shows how NOTCH pathway could be involved in the development of fibrosis. Using in this case the N1ICD overexpressing 12Z cells, we observed at transcriptional level, significantly increased the expression of fibrosis mediators TGF-β1, TGF-β2 and CTGF (Figure5.4A). Following that, normal endometrial stromal cells were treated with the conditional media (CM) from N1ICD overexpressing 12Z cells and showed significantly higher expression of α-SMA and COL1A1 compared to the CM from control 12Z cells (Figure 5.4B). This suggests that there could be other mechanisms that participate in the development of fibrosis in endometriosis that are worth investigating.

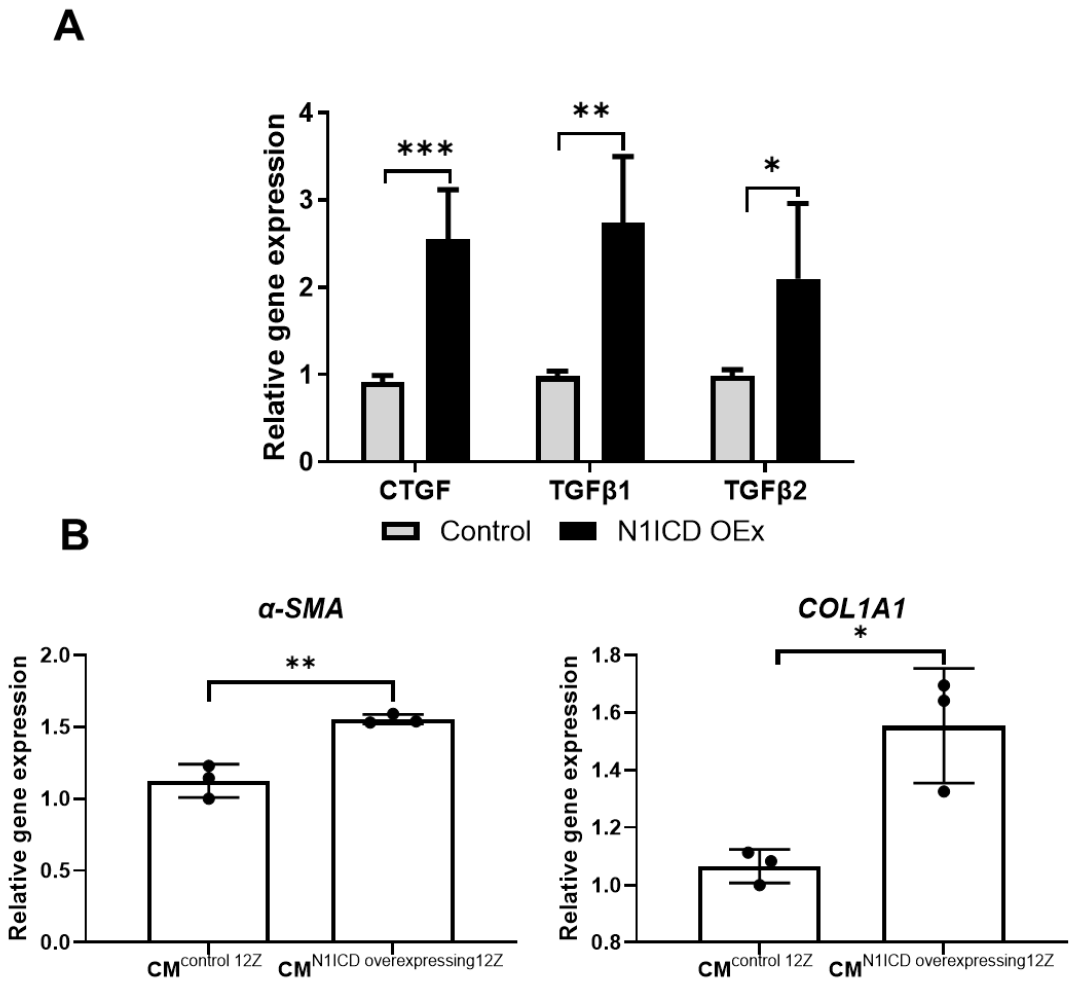

Figure 5.4: Conditional Media from N1ICD Overexpressing 12Z Cells Significantly Increases α-SMA and COL1A1 Expression in Endometrial Stromal Cells. (A) Overexpression of N1ICD in 12Z cells significantly increased the expression of fibrosis mediators TGF-β1, TGF-β2 and CTGF. (B) Normal endometrial stromal cells treated with the conditioned media (CM) from N1ICD overexpressing 12Z cells had significantly higher expression of α-SMA and COL1A1 compared to the CM from control 12Z cells.

5.3.6 B cells

B cells are a small cell population in the endometrium which has not been the focus of extensive research. The results from this study highlighted the importance of including the role of B cells in future studies. The findings in this study are focused on the mouse model of endometriosis and do not reflect the full scenario of the disease. The mouse

model provides important insights into human B cells development and disease[351] but there is still more research needed to be done to determine if the findings in mice are relevant to in humans. Several studies of mouse and human B cells have been focused on B cell development and B cell precursor populations[352] but there are no sufficient studies that can clarify the role of B cells in endometriosis.

Further studies focused on these cells would help to explain the role of B cells during the development of endometriosis. The present study revealed that B cells could be a suitable candidate for new therapeutic strategies which could be open a different and less invasive approach for the treatment of women with endometriosis.

While these novel studies discovered new avenues for the role of miR-21 and fibrosis in the context of endometriosis, more work is needed to fully understand how miR-21 can modulate fibrosis. The search for new models such as spheroids or 3D systems that permit the study of the interactions between multiple cell lines and how they interact with the environment, have been shown to have great promise and may serve as useful models for future studies [353].

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APPENDIX 1

SUPPLEMENTARY DATA FOR CHAPTER 1

Table 1A: Advantages of the Baboon model to study Endometriosis.

References	Main Focus of the study	Year
DaRif et al.[354]	Bacterial peritonitis	1984
D' Hooghe et al.[355]	Evolution of spontaneous endometriosis	**1992**
Cornillie et al.[356]	Morphological characteristics of spontaneous endometriosis	**1992**
Shalev et al.[357]	Endometriosis and stromal tumor	**1992**
D' Hooghe et al.[358]	Retrograde menstruation	**1994**
D' Hooghe et al.[359]	Fecundity	**1994**
Braundmeier et al.[173]	Fecundity	**2009**
D' Hooghe et al.[360]	Retrograde menstruation	**1996**
D' Hooghe et al.[176]	The effects of immunosuppression	**1995**
D' Hooghe et al.[361]	Intrapelvic injection of menstrual endometrium	**1995**
D' Hooghe et al.[362]	Peritoneal fluid volume and steroid hormone concentration	**1995**
D' Hooghe et al.[363]	Spontaneous endometriosis	**1995**
D' Hooghe et al.[364]	Anti-endometrial lymphocytotoxicity and natural killer cell activity	**1995**
D' Hooghe et al.[365]	White blood cell and subpopulations in peripheral blood	**1996**
D' Hooghe et al.[366]	Development of spontaneous endometriosis	**1996**
D' Hooghe et al.[367]	Disease progression	**1996**
D' Hooghe et al.[368]	The cycle pregnancy rate	**1996**
D' Hooghe et al.[369]	Luteinized unruptured follicle syndrome	**1996**
D' Hooghe et al.[174]	Clinical relevance of the baboon as a model for endometriosis	**1997**
D' Hooghe et al.[370]	The effect of pregnancy on endometriosis	**1997**
D' Hooghe et al.[371]	Pelvic inflammation	**1999**
Strakova et al.[177]	Decidualization	**2000**
Strakova et al. [178]	Decidualization	2003
Cameo et al.[179]	Decidualization	2006
Strug et al.[180]	Decidualization	2016
Ochoa-Bernal et al.[372]	Decidualization	2020
D' Hooghe et al.[373]	Effect of menstruation and intrapelvic injection on inflammatory parameters	2001
Fazleabas et al.[171]	Baboon model of endometriosis	**2002**
Fazleabas et al.[374]	Baboon model of endometriosis	2004

Table 1A (cont'd)

Fazleabas[283]	Baboon model of endometriosis	2006
Kyama et al. [375]	Baboon model of endometriosis	2007
D' Hooghe et al[376]	Baboon model of endometriosis	2009
Nair et al.[377]	Baboon model of endometriosis	2016
Fazleabas et al.[378]	Endometrial function	**2002**
D' Hooghe et al.[18]	Retrograde menstruation	**2002**
D' Hooghe et al.[181]	Infertility/subfertility	**2003**
Hastings et al.[48]	Infertility/subfertility	2006
Fazleabas at al.[182]	Steroid receptor and aromatase expression	**2003**
Moore et al.[379]	Spontaneous ovarian tumors	**2003**
Barrier et al.[380]	Adenomyosis associated with endometriosis	**2004**
Barrier et al.[381]	Anti-tumor necrosis factor therapy	**2004**
Falconer et al. [183]	The effect of cycle stage, lymphocyte suppression and pregnancy on CA-125 levels in peritoneal fluid and serum	**2005**
D' Hooghe et al.[382]	Fundamental and preclinical research	**2004**
Gashaw et al.[184]	Expression of factor CYR61 (CCN1)	**2006**
Hastings et al.[383]	Estrogen Response	**2006**
Falconer et al. [384]	Efficacy of anti-TNF monoclonal antibody in reducing established endometriosis	**2006**
Jones et al. [385]	Changes in ultrastructure and glycosylation of endometrium	**2006**
D' Hooghe et al.[386]	Recombinant human TNFRSF1A (r-hTBP1)	**2006**
Kyama et al.[387]	Effect of recombinant human TNF-binding protein-1 and GnRH antagonist	**2006**
Afshar et al.[185]	Notch signaling pathway	**2007**
Afshar et al.[186]	Notch signaling pathway	2012
Su et al. [187]	Notch signaling pathway	2015
Song et al.[99]	Notch signaling pathway	2020
Su at al.[188]	Notch signaling pathway	2016
Barrier et al.[388]	Ileocaecal junction and associated regional lymph nodes	**2007**
Kim et al.[389]	Expression of HOXA10	**2007**
Lebovic et al.[390]	PPAR-gamma receptor ligand	**2007**
Falconer et al.[391]	Effects of anti-TNF-mAb treatment on pregancy	**2008**
Bennet et al.[392]	Endometrial and cervical polyps	**2009**
Kyama et al.[189]	Role of cytokines	**2009**
Jones et al. [393]	Ultrastructure of ectopic peritoneal lesions	**2009**
Winterhager et al.[394]	Connexin expression pattern	**2009**
Banerjee et al.[395]	Implantation	**2010**
Fazleabas[60]	Progesterone resistance	**2010**
Joshi et al. [53]		2017
Sherwin et al.[396]	Endometrial response to chorionic gonadotropin	**2010**

Table 1A (cont'd)

Hapangama et al.[397]	Expression of regulators of cell-fate	**2010**
Ilad et al.[398]	Study of the ubiquitin-nuclear factor-Kb pathway	**2010**
Braundmeier et al.[399]	Extracellular matrix metalloproteinases	**2010**
Brosens et al.[400]	Proteomic analysis of endometrium	**2010**
Lebovic et al.[401]	Peroxisome proliferator-activated receptor-(gamma) receptor ligand	**2010**
Hey-Cunningham et al.[402]	Lymph nodes	**2011**
Morris et al.[403]	Changes in menstrual cycle	**2011**
Kemnitz et al.[404]	Calorie restriction and aging	**2011**
Harirchian et al.[405]	Lesion Kinetics	**2012**
Campo et al.[406]	Adenomyosis and infertility	**2012**
Afshar et al.[59]	Changes in eutopic endometrial gene expression	**2013**
Langoi et al.[407]	Aromatase inhibitor treatment	**2013**
Jagirdar et al.[408]	Pleuro-pulmonary endometriosis	**2013**
Donnez et al.[409]	Mimic human deep nodular lesions	**2013**
Donnez et al.[410]	Nerve fiber density in deep nodular endometriotic lesions	**2013**
Orellana et al.[411]	Nerve fiber density in deep nodular endometriotic lesions	2017
Giuliani et al.[94]	Characterization of uterine NK	**2014**
Kyama et al.[412]	Endometriosis induction	**2014**
Sugihara et al.[413]	Pro-apoptotic peptides as potential therapy	**2014**
Fazleabas at al.[86]	Changes in regulatory T cells	**2015**
Braundmeier et al.[414]	Changes in regulatory T cells	2012
Joshi et al.[147]	MicroRNAs	**2015**
Nothnick et al. [192]	MicroRNAs	2017
Kim et al.[190]	STAT3	**2015**
Yoo et al.[313]	STAT3	2016
Su et al. [415]	Implantation and establishment of pregnancy	**2015**
Baumann et al. [416]	Arginine methyltransferases	**2015**
Zhang et al.[118]	Epithelial-Mesenchymal-Transition (EMT)	**2016**
Parkin et al.[417]	Uterine Leukocyte Function and Dysfunction	**2016**
Slayden[418]	Translational in vivo models for women's health	**2016**
Hussein et al.[419]	c-Jun NH2-terminal kinase inhibitor bentamapimod	**2016**
Taylor et al.[191]	Effect of simvastatin	**2017**
Cosar et al.[193]	Effect of simvastatin	2019
Yoo et at.[420]	KRAS activation and overexpression of SIRT1/BCL6	2017
Stouffer et al.[421]	A vital model for female reproduction	2017
Drury et al. [422]	Changes in uterine NK	**2018**
Chang et al.[423]	Overexpression of Four Joint Box-1 Protein (FJX1)	**2018**

Table 1A (cont'd)

Hufnagel et al. [424]	Icon immunoconjugate treatment	**2018**
Nothnick et al.[425]	Expression of Macrophage Migration Inhibitory Factor Receptor	**2018**
Kim et al.[426]	HDAC3 and infertility	**2019**
Hapangama et al.[427]	Endometriosis associated with abnormally located endometrial basalis-like (SSEA1+/SOX9+	**2019**
Kirejczyk et al.[428]	Urogenital lesions	2021
Le et al.[429]	Effects of endometriosis on immunity and mucosal microbial community dynamics	2022
Poirier et al.[430]	Inhibition of estradiol synthesis	2022

APPENDIX 2

SUPPLEMENTARY DATA FOR CHAPTER 2

Table 2A: Genes expressed involved in connective tissue disorders.

Categories	**Disease or Functions**	**Genes involved**
Connective Tissue Disorders, Organismal Injury and Abnormalities, Tissue Morphology	Degeneration of connective tissue	C5,CD28,CFD,CYTL1,DEPP1,FGF18,GABRB3,GRID1,IL10,IL1B,IL1RL1,IL1RN,IL33,IL6,Masp1,MKX,MMP10,MMP13,MMP3,NDP,OSM,PRKCQ,TNFSF11,TP63
Connective Tissue Disorders, Organismal Injury and Abnormalities	Collagen disease	ADIPOQ,BGN,CEBPA,CFD,CIDEC,COL12A1,COL7A1,COL8A1,F10,FST,G0S2,HMGCS2,IGFN1,KLB,KLHL34,LEP,MLF1,PLIN1,PNPLA3,PPARG,RBP4,TNFSF11,TRIM63
Connective Tissue Disorders, Organismal Injury and Abnormalities	Abnormality of adipose tissue	ABCA1,ABCG1,ADIPOQ,ADRB3,CCR7,CD36,CD5L,CIDEC,CRHR2,CYP26A1,FABP4,Fam13a,FGF10,GDF3,HSD11B1,IL33,Irs3,LEF1,LEP,LIPA,LPL,OSM,PLIN1,PPARG,SCG5,STEAP4,UCP3
Connective Tissue Disorders, Organismal Injury and Abnormalities, Tissue Morphology	Deterioration of connective tissue	ADRB3,C5,CD28,CFD,CLEC5A,CXCL3,CYBB,CYTL1,DEPP1,F10,FGF18,FST,GABRB3,IHH,IL10,IL1B,IL1RL1,IL1RN,IL33,IL6,Masp1,MMP10,MMP13,MMP3,OSM,PRKCQ,SCN1B,SLC6A2,TF,TNFSF11,TP63,VDR
Connective Tissue Disorders, Organismal Injury and Abnormalities	Damage of connective tissue	APOE,C5,CD28,CFD,CYTL1,DEPP1,FGF18,IL10,IL1B,IL1RL1,IL1RN,IL33,IL6,Masp1,MMP10,MMP13,MMP3,OSM,PRKCQ,TNFSF11,TP63

Table 2B: Genes expressed involved in inflammatory diseases.

Categories	Disease or Functions	Genes involved
Connective Tissue Disorders, Inflammatory Disease, Inflammatory Response, Organismal Injury and Abnormalities, Skeletal and Muscular Disorders	Inflammation of joint	ACSL1,ACSL6,ACTA1,ADAMTS16,ADAMTS17,ADAMTS18,ADAMTS20,ADAMTS7,ADGRA1,ADIPOQ,ADRB3,AHSG,ALDOA,APOE,AQP9,BGN,C5,C6,CCL17,CCL22,CCL24,CCL3L3,Ccl6,Ccl9,CCR7,CCRL2,CD200R1,CD28,CD36,CD3D,CD3E,CD4,CD6,CD79A,CD83,CDKN2A,CFD,CLEC4D,CLEC4E,CLEC5A,CNR1,COCH,COL11A1,COLQ,COMP,CSF2RB,CSF3R,CTLA4,CXCL14,CXCL3,Cxcl9,CXCR4,CYTL1,DEPP1,EDN3,ENPP2,F10,F7,FABP4,FGF10,FGF18,FGR,FKBP5,FMOD,FPR2,G0S2,GABRB3,GZMA,HAVCR2,HLA,HP,IGHG1,IGHM,IGKC,Igkv1-117,IL10,IL1B,IL1RL1,IL1RN,IL2RB,IL33,IL6,IL7R,IP6K3,ITGB2,KCNJ15,L3MBTL4,LEP,LYZ,MAPT,Masp1,MC2R,MEFV,MME,MMP10,MMP12,MMP13,MMP2,MMP27,MMP3,MS4A1,MYBPC2,NCF4,NR1H3,NR4A3,NXPE3,OSM,PDCD1,PDGFRL,PGLYRP2,PPARG,PRDM1,PRKCQ,PROCR,PTGER2,PTX3,RAD51B,RBP4,RCSD1,RETN,RGCC,RUNX2,S100A8,S100A9,Saa3,SCN1B,SCN4A,SCN4B,SELE,SELP,SH2D2A,SLC11A1,SLC1A3,SLC6A2,SOD3,STEAP4,SYNGR1,TF,TIMP4,TMEFF2,TNFSF11,TNNC1,UBASH3A,UNC93A,VDR,VIPR2,WNT11
Connective Tissue Disorders, Inflammatory Disease, Organismal Injury and Abnormalities, Skeletal and Muscular Disorders	Rheumatic Disease	ACSL1,ACSL6,ACTA1,ADAMTS16,ADAMTS17,ADAMTS18,ADAMTS20,ADAMTS7,ADGRA1,ADIPOQ,ADRB3,AHSG,ALDOA,APOE,AQP9,BGN,C5,C6,CAMK2B,CCL17,CCL22,CCL24,CCL3L3,Ccl6,Ccl9,CCR7,CCRL2,CD19,CD200R1,CD28,CD36,CD3D,CD3E,CD4,CD6,CD79A,CD83,CDKN2A,CFD,CLEC4D,CLEC4E,CLEC5A,CNR1,COCH,COL11A1,COL9A2,COLQ,COMP,CSF2RB,CSF3R,CTLA4,CXCL14,CXCL3,Cxcl9,CXCR4,CYTL1,DEPP1,EDN3,EDNRB,ENPP2,F10,F7,FABP4,FFAR2,FGF10,FGF18,FGR,FKBP5,FMOD,FPR2,G0S2,GABRB3,GFI1,GZMA,HAVCR2,HLA-A,HP,IGHG1,IGHM,IGKC,Igkv1-117,IL10,IL1B,IL1RL1,IL1RN,IL2RB,IL33,IL6,IL7R,IP6K3,ITGB2,KCNJ15,KCNJ2,L3MBTL4,LEP,LYZ,MAPT,Masp1,MC2R,MEFV,MME,MMP10,MMP12,MMP13,MMP2,MMP27,MMP3,MS4A1,MYBPC2,NCF4,NR1H3,NR4A3,NXPE3,OSM,PDCD1,PDGFRL,PGLYRP2,PPARG,PRDM1,PRKCQ,PROCR,PTGER2,PTX3,RAD51B,RBP4,RCSD1,RETN,RGCC,RUNX2,S100A8,S100A9,Saa3,SCN1B,SCN4A,SCN4B,SELE,SELP,

Table 2B (cont'd)

Inflammatory Disease, Organismal Injury and Abnormalities	Chronic inflammatory disorder	SFRP4,SH2D2A,SLC11A1,SLC1A3,SLC6A2,SOD3, STEAP4,SYNGR1,TF,THBS4,TIMP4,TLR8,TMEFF2, TNFSF11,TNNC1,UBASH3A,UNC93A,VDR,VIPR2, WNT11,ZBTB16ABCA1,ABCB4,ABCG1,ACSL1,ACT A1,ADAMTS7,ADGRA1,ADIPOQ,ADORA1,ADRB3,A HSG,ALDOA,AMY2A,ANK1,APOE,AQP9,BACH2,BG N,C5,CA3,CA4,CASR,CCL17,CCL22,CCL24,Ccl6,Cc l9,CCR7,CCRL2,CD19,CD28,CD36,CD3D,CD4,CD6, CD79A,CD83,CDKN2A,CEBPA,CFD,CHRM2,CKM,C LEC4D,CLEC4E,COCH,COLQ,COMP,CRYBB1,CSF 2RB,CSF3R,CTLA4,CXCL14,CXCL3,Cxcl9,CXCR4, CYBB,CYP2E1,DNAH5,EDN3,EDNRB,F10,FABP4,F GF10,FGR,FKBP5,FPR2,G0S2,GABRB3,GPNMB,G ZMA,H2-Eb2,HAVCR2,HLA-A,HP,HSD11B1,IGHG1,IGHM,IGKC,IL10,IL13RA2,IL 1B,IL1RL1,IL1RN,IL2RB,IL33,IL6,IL7R,IP6K3,ITGB2, KCNA2,KCNA5,KCNA6,KCNA7,KCNB2,KCNC1,KCN J15,L3MBTL4,LEP,LMCD1,LRP2,LYZ,Masp1,MC2R, MEFV,MME,MMP13,MMP2,MMP3,MPEG1,MS4A1, MYBPC2,NCF4,NR1H3,NR4A3,NXPE3,OSM,PBLD, PDCD1,PDGFRL,PPARG,PRDM1,PRKCQ,PROCR, PTGER2,RAD51B,RBP4,RCSD1,RGCC,S100A8,S10 0A9,Saa3,SCN4A,SELE,SELP,SERPINA3,SH2D2A, SLC1A3,SLC6A2,SLPI,STAC,STEAP4,STRA6,SYNG R1,TF,TLR8,TNFRSF13C,TNFRSF17,TNFSF11,TNN C1,TRIM7,UBASH3A,UNC93A,VDR,VIPR2,WNT11
Connective Tissue Disorders, Immunological Disease, Inflammatory Disease, Inflammatory Response, Organismal Injury and Abnormalities, Skeletal and Muscular Disorders	Rheumatoid arthritis	ACSL1,ACTA1,ADAMTS7,ADGRA1,ADIPOQ,AHSG, ALDOA,APOE,AQP9,BGN,C5,CCL17,CCL22,CCL24, Ccl6,Ccl9,CCR7,CCRL2,CD28,CD36,CD3D,CD4,CD 6,CD79A,CD83,CFD,CLEC4D,CLEC4E,COCH,COL Q,COMP,CSF2RB,CSF3R,CTLA4,CXCL14,CXCL3,C XCR4,EDN3,F10,FABP4,FGF10,FKBP5,FPR2,G0S2, GABRB3,GZMA,HAVCR2,HLA-A,HP,IGHG1,IGHM,IGKC,IL10,IL1B,IL1RN,IL2RB,IL3 3,IL6,IL7R,IP6K3,ITGB2,KCNJ15,L3MBTL4,LYZ,Mas p1,MC2R,MEFV,MME,MMP13,MMP2,MMP3,MS4A1, MYBPC2,NCF4,NR1H3,NR4A3,NXPE3,OSM,PDCD1 ,PDGFRL,PPARG,PRDM1,PTGER2,RAD51B,RBP4, RCSD1,RGCC,S100A8,S100A9,Saa3,SCN4A,SELE, SH2D2A,SLC6A2,STEAP4,SYNGR1,TNFSF11,TNN C1,UBASH3A,UNC93A,VDR,VIPR2,WNT11

Table 2C: Genes expressed involved in inflammatory responses.

Categories	Disease or Functions	Genes involved
Connective Tissue Disorders, Inflammatory Disease, Inflammatory Response, Organismal Injury and Abnormalities, Skeletal and Muscular Disorders	Inflammation of joint	ACSL1,ACSL6,ACTA1,ADAMTS16,ADAMTS17,ADAMTS18,ADAMTS20,ADAMTS7,ADGRA1,ADIPOQ,ADRB3,AHSG,ALDOA,APOE,AQP9,BGN,C5,C6,CCL17,CCL22,CCL24,CCL3L3,Ccl6,Ccl9,CCR7,CCRL2,CD200R1,CD28,CD36,CD3D,CD3E,CD4,CD6,CD79A,CD83,CDKN2A,CFD,CLEC4D,CLEC4E,CLEC5A,CNR1,COCH,COL11A1,COLQ,COMP,CSF2RB,CSF3R,CTLA4,CXCL14,CXCL3,Cxcl9,CXCR4,CYTL1,DEPP1,EDN3,ENPP2,F10,F7,FABP4,FGF10,FGF18,FGR,FKBP5,FMOD,FPR2,G0S2,GABRB3,GZMA,HAVCR2,HLA-A,HP,IGHG1,IGHM,IGKC,Igkv1-117,IL10,IL1B,IL1RL1,IL1RN,IL2RB,IL33,IL6,IL7R,IP6K3,ITGB2,KCNJ15,L3MBTL4,LEP,LYZ,MAPT,Masp1,MC2R,MEFV,MME,MMP10,MMP12,MMP13,MMP2,MMP27,MMP3,MS4A1,MYBPC2,NCF4,NR1H3,NR4A3,NXPE3,OSM,PDCD1,PDGFRL,PGLYRP2,PPARG,PRDM1,PRKCQ,PROCR,PTGER2,PTX3,RAD51B,RBP4,RCSD1,RETN,RGCC,RUNX2,S100A8,S100A9,Saa3,SCN1B,SCN4A,SCN4B,SELE,SELP,SH2D2A,SLC11A1,SLC1A3,SLC6A2,SOD3,STEAP4,SYNGR1,TF,TIMP4,TMEFF2,TNFSF11,TNNC1,UBASH3A,UNC93A,VDR,VIPR2,WNT11
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Cell movement of phagocytes	ABCA1,ABCB4,ACKR1,ADIPOQ,ADORA1,ADRB3,APOE,AQP9,C5,C6,CALCB,CASR,CCL17,CCL22,CCL24,CCL3L3,Ccl9,CCR3,CCR7,CCRL2,CD28,CD300A,CD36,CD4,CEBPE,CFD,CLEC4M,CNR1,CSF2RB,CSF3R,CXCL14,CXCL3,Cxcl3,CXCR4,CYBB,CYP26A1,CYTL1,Ear2 (includes others),EDIL3,EDN3,EDNRB,EEF1A2,F10,F2R,F7,FABP4,FFAR2,FGR,FPR2,GREM1,Gzmb,HP,Ighg2b,IL10,IL1B,IL1RL1,IL1RN,IL33,IL34,IL6,ITGAD,ITGAX,ITGB2,KLF15,LEP,LILRB3,MAPT,MARCO,Masp1,Mcpt4,mir-133,MMP10,MMP12,MMP2,MS4A4A,MYO1F,NOS1,NR1H3,OSM,PBLD,PDCD1,PDK2,PDK4,PLA2G7,PLTP,PPARG,PRDM1,PRKCQ,PROCR,PTPRO,PTX3,RETN,S100A8,S100A9,SELE,SELP,SERPINA3,SFRP1,SFRP5,SLAMF8,SLPI,SOD3,THBS4,TNFSF11,TREM2

Table 2C (cont'd)

Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	Activation of leukocytes	ADIPOQ,ADORA1,APOE,BACH2,BTLA,C5,C6,CASR,CCL17,CCL22,CCR7,CD19,CD200R1,CD22,CD28,CD300A,CD300LB,CD300LD,CD36,CD3E,CD4,CD6,CD79A,CD83,CD84,CD8A,CD8B,CEBPA,CEBPE,CHRNA4,CLEC4D,CLEC4E,CLEC4M,CLEC5A,CLEC7A,CNR1,CSF2RB,CTLA4,CXCL3,CYBB,F10,F7,FCRL1,FPR2,GFI1,GPNMB,GPR137B,GZMA,Gzmb,HAVCR2,HLA-A,Hoxa11os,HSD11B1,HVCN1,Igha,IGHG1,IGHM,Igkv1-117,IL10,IL13RA2,IL1B,IL1RL1,IL1RN,IL2RB,IL33,IL34,IL6,ITGAD,ITGB2,KLRB1,LEP,LILRB3,LILRB4,MAOB,MAPT,MME,MPZ,MRGPRX2,MS4A1,NR1H3,NR4A3,Orm1 (includes others),OSM,PDCD1,PDK2,PDK4,PGLYRP2,PILRB,POU2AF1,PPARG,PRDM1,PRF1,PRG2,PRKCQ,PTGER2,RBP4,Retnla,S100A8,S100A9,Saa3,SELE,SELP,SFRP1,SH2D2A,SIGLEC8,SLC11A1,SLPI,SUCNR1,TLR8,TNFRSF13C,TNFRSF17,TNFSF11,TREM2,VIPR2
Inflammatory Response	Inflammatory response	ABCD2,ACKR1,ACOD1,ADIPOQ,ADORA1,AHSG,APOE,AQP9,ART3,C2CD4A,C5,C6,CALCB,CASR,CCL17,CCL22,CCL24,CCL3L3,CCR3,CCR7,CCRL2,CD28,CD300A,CD36,CD4,CD84,CD96,CDO1,CEBPA,CEBPE,CLEC4E,CLEC4M,CLEC5A,CLEC7A,CSF3R,Ctla2a/Ctla2b,CXCL14,CXCL3,Cxcl3,Cxcl9,CXCR4,CYBB,CYTL1,Ear2 (includes others),EDN3,EDNRB,F2R,F7,FABP4,FFAR2,FGR,FPR2,GLI1,GPR137B,GPRC5B,GREM1,HAVCR2,HLA-A,Hoxa11os,HP,IGHG1,IGHM,IL10,IL1B,IL1RL1,IL1RN,IL33,IL34,IL6,ITGB2,KLRG1,LEP,LILRA5,LILRB3,LILRB4,LIPA,LPL,LYZ,MAOB,MAPT,MARCO,MEFV,MME,MMP2,MYO1F,NDP,NOS1,NOX4,NR1H3,OLR1,Orm1 (includes others),OSM,PDCD1,PDK2,PDK4,PGLYRP2,PLA2G7,PPARG,PRDM1,PRF1,PRG2,PROCR,PTAFR,PTGER2,PTPRO,PTX3,Retnla,Retnlg,S100A8,S100A9,SELE,SELP,SERPINA3,SFRP1,SFRP5,SLAMF8,SLC11A1,SLPI,SOD3,SUCNR1,THBS4,TLR8,TNFSF11,TREM2,ZBTB16

Table 2D: Genes expressed involved in cancer.

Categories	Disease or Functions	Genes involved
Cancer, Organismal Injury and Abnormalities	Non-hematological solid tumor	A4GALT,AACS,ABCD3,ABCE1,ABHD11,ABHD13,ABHD6,ABI1,ACAT2,ACOT12,ACP1,ACSL3,ACTG1,ACTL6A,ACTR2,ACVR1B,ADAM10,ADAMTS3,ADGB,AGPAT2,AK2,ALAS1,ALDH18A1,ALG14,ALG3,ALG8,ALG9,AMD1,ANKRD13A,ANKRD46,AP1G1,AP1S1,AP1S2,AP2M1,AP3M1,APOBEC3B,AQP3,AQR,ARAP2,ARCN1,ARF1,ARF4,ARF6,ARFGEF1,ARFGEF2,ARHGAP12,ARHGAP32,ARHGAP39,ARPC1A,ARPC5,ARRDC4,ASAH1,ASIC2,ATAD3A,ATIC,ATP1B3,ATP4A,ATP6V0A2,ATP6V1A,AZIN1,B3GALT6,B4GALT3,BACE2,BAIAP2L1,BAK1,BARX2,BAZ1A,BBLN,BGLAP,BLCAP,BOP1,BRIX1,BTBD10,BTBD9,BUD23,BYSL,BZW1,BZW2,C11orf52,C1GALT1,C1orf226,C2CD2L,C3,C9orf64,CA5B,CAB39,CACNA2D3,CALML3,CAPN7,CAPSL,CBX4,CCDC116,CCDC25,CCDC42,CCDC65,CCDC86,CCDC88C,CCT3,CCT6A,CD2AP,CD55,CDA,CDC42SE1,CDIPT,CDV3,CEACAM19,CFAP221,CFAP52,CFB,CHD9,CHFR,CHML,CHMP2B,CHMP3,CHMP4B,CHST11,CHUK,CIAPIN1,CIPC,CISH,CLCN3,CLIC1,CLINT1,CLPTM1L,CLTC,CMPK1,CMTM6,CNBD2,COG3,COMMD10,COMMD7,COPB1,COPB2,COPZ1,CPN1,CPSF2,CRK,CRLF1,CRLS1,CROCC,CTBS,CTNNAL1,CTNND1,CTSV,CTU2,CXADR,CYB561,CYB561D2,CYP2J2,CYP51A1,CYSRT1,DAD1,DBNL,DCAF13,DCLRE1B,DDX1,DDX10,DDX21,DDX31,DDX39A,DDX3X,DDX54,DDX56,DENND2C,DENND2D,DENR,DHCR24,DHCR7,DHX32,DNAJC11,DNAJC17,DNAJC2,DNAJC3,DNTTIP2,DOK4,DOLPP1,DPAGT1,DPP3,DPY19L1,DR1,DSEL,ECE1,EDEM3,EEA1,EEF1E1,EFL1,EIF1AX,EIF2B5,EIF2S1,EIF2S2,EIF3B,EIF3D,EIF4A1,EIF4A3,EIF4E,EIF4G1,EIF5A,EIF5B,EIF6,ELOVL1,ELOVL6,ENDOD1,ENOPH1,ENTPD6,EPB41L4B,EPRS1,EPS8,ERAP1,ERG28,ERH,ERLIN2,ERO1A,ESCO1,ETF1,ETFDH,EXOC8,EXOSC1,EZR,F11R,F2RL1,F8A1 (includes others),FAM136A,FAM3C,FANK1,FAR1,FARSA,FARSB,FBXO28,FDFT1,FDPS,FGFRL1,FLNB,FOXN2,FPGS,FPGT,FRG1,FRRS1,FSCN2,FUCA1,FURIN,FZD5,G3BP1,G6PD,GAK,GAL,GALE,GALNT4,GALNT7,GCH1,GCK,GDPD1,GFER,GFPT1,GFRA4,GIPC1,GMDS,GMPPB,GNG12,GNG4,GOLGA1,GOLGA7,GORASP2,GPATCH4,GPD1L,GPD2,GPR151,GPS1,GPX1,

Table 2D (cont'd)

		GRPEL2,GRWD1,GSPT1,GSS,GSTO1,GTF2F2HSD17B12,HSD17B7,HSPA4,HYOU1,IBTK,IDI1,IER3,IFT43,IL17B,IL1A,IL1R1,IL24,IL36A,IL36B,INSIG1,IPO5,IQGAP2,ISYNA1,ITGA6,ITPA,JAGN1,JPT1,JPT2,JUP,KAT14,KAZN,KBTBD8,KCNF1,KCNK5,KIAA1217,KIAA2013,KIF16B,KIF21A,KIF5B,KIF5C,KLF3,KLF4,KLK11,KLK3,KPNB1,KREMEN2,KRT4,KRTCAP3,KYAT1,L2HGDH,LAMB3,LARP1,LARP4,LDLR,LENG1,LIF,LIN7C,LIPH,LIPK,LNX2,LONRF3,LPCAT4,LPO,LRBA,LRG1,LRP8,LRRC41,LRRC59,LRRC71,LRRC8E,LSR,LSS,LTF,LTV1,LURAP1L,Ly6a (includes others), LY6D,LYAR,M6PR,MACIR,MAGOH,MAK16,MAN1A2,MANBA,MANSC1,MAP2K1,MAP2K4,MAP3K1,MAP6,MAPK6,MAPKAPK3,MAPRE1,MAT2A,MATCAP2,MBOAT2,MBTPS2,MDFI,MEA1,MED9,MET,METAP1,METTL1,MFSD2A,MFSD6,MGAT2,MICALL2,MID2,MIF4GD,MLX,MOGAT1,MORF4L2,MPDU1,MPHOSPH10,MPHOSPH6,MPZL3,MRPL17,MRPL19,MRPL20,MRPL36,MRPL46,MRPL52,MRPS10,MRPS18B,MRTO4,MSANTD1,MSL2,MSMO1,MST1R,MTA3,MTUS1,MTX2,MUC20,MVD,MVK,MYH14,MYL7,NAA15,NAA25,NAAA,NADK,NARS2,NAT10,NBAS,NCKAP1,NCSTN,NDFIP2,NDUFA11,NECTIN4,NELFE,NEPRO,NEU1,NHP2,NIFK,NIP7,NIPA2,NIPAL2,NIPSNAP3A,NKRF,NMD3,NME1,NOL11,NOL4L,NOL9,NOMO1 (includes others),NOP16,NOP2,NOP56,NOP58,NPSR1,NRTN,NSUN2,NTN1,NUCB1,NUDCD1,NUDCD2,NUMB,NUP50,NUP58,NUS1,OARD1,OSBPL3,OSBPL7,OSTC,PA2G4,PAG1,PAK1IP1,PAK2,PANK3,PAPLN,PAXBP1,PCLO,PCOLCE2,PCYT2,PDCD6,PDCD6IP,PDE12,PDZK1,PEF1,PEMT,PEPD,PES1,PEX2,PGAM5,PGLYRP1,PHF14,PHLDA1,PIGU,PIM1,PIP4K2C,PLEKHA7,PLEKHB2,PLEKHG3,PLEKHM1,PLET1,PLPP2,PLPP5,PLSCR1,PMEL,PMM2,PMVK,PNO1,PNPO,POLR2F,POLR3D,POLR3E,PON3,PPA1,PPFIA1,PPIP5K2,PPM1G,PPP1R10,PPP1R11,PPP1R14B,PPP1R15B,PPP2R1B,PPP2R2C,PPRC1,PREP,PRKAB1,PRKCE,PRMT7,PROM1,PRPF31,PRPF40A,PRR15,PSMA6,PSMB2,PSMB5,PSMD1,PSME3,PTBP3,PTK2,PTPRJ,PUM3,PUS1,PWWP2A,PYCR2,QRSL1,RAB11FIP1,RAB27B,RAB5IF,RAB6B,RAD23B,RALA,RALBP1,RAN,RANGAP1,RARS2,

Table 2D (cont'd)

		RASD1,RASGRP1,RBBP8,RBIS,RBM3,RBM34,RBM45,RBM47,RBP2,RCC1,REEP3,REL,RELCH,RER1,RET,RGP1,RIN1,RIOX2,RNF149,RNF17,RNF19A,RNF224,RNF7,RPF1,RPF2,RPH3AL,RPL7L1,RPN1,RRAS2,RRP12,RRP15,RRS1,RSPH3,RWDD4,RYBP,SC5D,SCAMP2,SCFD1,SCFD2,SCO1,SCRIB,SCTR,SDAD1,SDCBP,SEC11C,SEC13,SEC24C,SEC61A1,SEC61B,SECISBP2,SEL1L3,SELENOI,SEPHS2,SERBP1,SERP1,SETX,SF3A3,SFXN1,SH3BGRL2,SHISA2,SHOC2,SKIC3,SLC10A3,SLC10A7,SLC16A1SLC16A6,SLC1A5,SLC20A1,SLC25A24,SLC30A7,SLC31A1,SLC33A1,SLC34A2,SLC34A3,SLC35A3,SLC35C1,SLC39A11,SLC39A9,SLC52A2,SLC7A1,SLC7A6OS,SLC7A7,SLCO2A1,SLCO4A1,SLCO5A1,SLITRK4,SLK,SMPDL3A,SMU1,SNAPC1,SNAPC2,SNRNP40,SNRPD1,SNU13,SNX7,SOWAHC,SOX9,SPCS2,SPECC1,SPOCK1,SPRTN,SPTLC1,SPTLC2,SPTY2D1,SQLE,SREBF2,SRM,SRP19,SRP9,SRPRB,SRSF2,SSB,SSH3,SSR1,SSR2,SSR3,SSU72,SSX2IP,STAP2,STARD4,STAT5A,STC2,STEAP1,STEAP2,STIM2,STRAP,STRIP1,STT3A,STT3B,STX19,SULF2,SWSAP1,SYPL1,TAF5L,TAPT1,TAX1BP3,TBL3,TBRG4,TCERG1,TDG,TENT5B,TEX2,TGM2,THG1L,THSD4,THYN1,TIAM1,TICAM1,TINAGL1,TIPARP,TLCD3A,TLNRD1,TM9SF2,TM9SF3,TMED10,TMED2,TMED7,TMED9,TMEM128,TMEM14C,TMEM158,TMEM164,TMEM183A,TMEM190,TMEM238,TMEM248,TMEM30B,TMEM41A,TMEM41B,TMEM63A,TMEM68,TMEM87B,Tmsb4x (includes others), TNFAIP8L1,TNS4,TOMM40,TOMM70,TOR2A,TPD52,TPD52L1,TRABD,TRAF4,TRIB2,TRIM2,TRIP11,TRMT61A,TRPC3,TSFM,TSG101,TSR1,TTC13,TTC27,TTC39C,TUBA1C,TUBB4B,TWF1,TXNDC17,TXNDC9,TXNRD1,UAP1,UBA5,UBA6,UBQLN1,UBXN2A,UBXN2B,UCHL3,UCK2,UFL1,UNC13B,URB2,USO1,USP16,USP4,UTP14A,UTP14C,UTP18,UTP20,UXS1,VDAC2,VOPP1,VPS53,WAPL,WDR1,WDR36,WDR43,WDR75,WFS1,WIPI1,WWP2,XPNPEP1,XPOT,YIPF2,YIPF6,YKT6,YRDC,YWHAQ,ZBTB42,ZCCHC9,ZDHHC21,ZDHHC5,ZDHHC9,ZFP92,ZNF672,ZNF800

Table 2D (cont'd)

Cancer, Organismal Injury and Abnormalities	Nonhematologic malignant neoplasm	A4GALT,AACS,ABCD3,ABCE1,ABHD11,ABHD13,ABHD6,ABI1,ACAT2,ACOT12,ACP1,ACSL3,ACTG1,ACTL6A,ACTR2,ACVR1B,ADAM10,ADAMTS3,ADGB,AGPAT2,AK2,ALAS1,ALDH18A1,ALG14,ALG3,ALG8,ALG9,AMD1,ANKRD13A,ANKRD46,AP1G1,AP1S1,AP1S2,AP2M1,AP3M1,APOBEC3B,AQP3,AQR,ARAP2,ARCN1,ARF1,ARF4,ARF6,ARFGEF1,ARFGEF2,ARHGAP12,ARHGAP32,ARHGAP39,ARPC1A,ARPC5,ARRDC4,ASAH1,ASIC2,ATAD3A,ATIC,ATP1B3,ATP4A,ATP6V0A2,ATP6V1A,AZIN1,B3GALT6,B4GALT3,BACE2,BAIAP2L1,BAK1,BARX2,BAZ1A,BBLN,BGLAP,BLCAP,BOP1,BRIX1,BTBD10,BTBD9,BUD23,BYSL,BZW1BZW2,C11orf52,C1GALT1,C1orf226,C2CD2L,C3,C9orf64,CA5B,CAB39,CACNA2D3,CALML3,CAPN7,CAPSL,CBX4,CCDC116,CCDC25,CCDC42,CCDC65,CCDC86,CCDC88C,CCT3,CCT6A,CD2AP, CD55,CDA,CDC42SE1,CDIPT,CDV3,CEACAM19,CFAP221,CFAP52,CFB,CHD9,CHFR,CHML,CHMP2B,CHMP3,CHMP4B,CHST11,CHUK,CIAPIN1,CIPC,CISH,CLCN3,CLIC1,CLINT1,CLPTM1L,CLTC,CMPK1,CMTM6,CNBD2,COG3,COMMD10,COMMD7,COPB1,COPB2,COPZ1,CPN1,CPSF2,CRK,CRLF1,CRLS1,CROCC,CTBS,CTNNAL1,CTNND1,CTSV,CTU2,CXADR,CYB561,CYB561D2,CYP2J2,CYP51A1,CYSRT1,DAD1,DBNL,DCAF13,DCLRE1B,DDX1,DDX10,DDX21,DDX31,DDX39A,DDX3X,DDX54,DDX56,DENND2C,DENND2D,DENR,DHCR24,DHCR7,DHX32,DNAJC11,DNAJC17,DNAJC2,DNAJC3,DNTTIP2,DOK4,DOLPP1,DPAGT1,DPP3,DPY19L1,DSEL,ECE1,EDEM3,EEA1,EEF1E1,EFL1,EIF1AX,EIF2B5,EIF2S1,EIF2S2,EIF3B,EIF3D,EIF4A1,EIF4A3,EIF4E,EIF4G1,EIF5A,EIF5B,EIF6,ELOVL1,ELOVL6,ENDOD1,ENOPH1,ENTPD6,EPB41L4B,EPRS1,EPS8,ERAP1,ERG28,ERH,ERLIN2,ERO1A,ESCO1,ETF1,ETFDH,EXOC8,EXOSC1,EZR,F11R,F2RL1,F8A1 (includes others), FAM136A,FAM3C,FANK1,FAR1,FARSA,FARSB,FBXO28,FDFT1,FDPS,FGFRL1,FLNB,FOXN2,FPGS,FPGT,FRG1,FRRS1,FSCN2,FUCA1,FURIN,FZD5,G3BP1,G6PD,GAK,GAL,GALE,GALNT4,GALNT7,GCH1,GCK,GDPD1,GFER,GFPT1,GFRA4,GIPC1,GMDS,GMPPB,GNG12,GNG4,GOLGA1,GOLGA7,GORASP2

Table 2D (cont'd)

		,GPATCH4,GPD1L,GPD2,GPR151,GPS1,GPX1,GRPEL2,GRWD1,GSPT1,GSS,GSTO1,GTF2F2,GTF3C6,GTPBP4,HCRTR1,HDAC3,HEATR1,HEATR5A,HEBP2,HESX1,HGH1,HIF1A,HIP1R,HM13,HMGCR,HMGCS1,HNRNPAB,HNRNPD,HNRNPF,HORMAD2,HSD17B11,HSD17B12,HSD17B7,HSPA4,HYOU1,IBTK,IDI1,IER3,IFT43,IL17B,IL1A,IL1R1,IL24,IL36A,IL36B,INSIG1,IPO5,IQGAP2,ISYNA1,ITGA6,ITPA,JAGN1,JPT1,JPT2,JUP,KAT14,KAZN,KBTBD8,KCNF1,KCNK5,KIAA1217,KIAA2013,KIF16B,KIF21A,KIF5B,KIF5C,KLF3,KLF4,KLK11,KLK3,KPNB1,KREMEN2,KRT4,KRTCAP3,KYAT1,L2HGDH,LAMB3,LARP1,LARP4,LDLR,LENG1,LIF,LIN7C,LIPH,LIPK,LNX2,LONRF3,LPCAT4,LPO,LRBA,LRG1,LRP8,LRRC41,LRRC59,LRRC71,LRRC8E,LSR,LSS,LTF,LTV1,LURAP1L,Ly6a (includes others),LY6D,LYAR,M6PR,MACIR,MAGOH,MAK16,MAN1A2,MANBA,MANSC1,MAP2K1,MAP2K4,MAP3K1,MAP6,MAPK6,MAPKAPK3,MAPRE1,MAT2A,MATCAP2,MBOAT2,MBTPS2,MDFI,MEA1,MED9,MET,METAP1,METTL1,MFSD2A,MFSD6,MGAT2,MICALL2,MID2,MIF4GD,MLX,MOGAT1,MORF4L2,MPDU1,MPHOSPH10,MPHOSPH6,MPZL3,MRPL17 MRPL19,MRPL20,MRPL36,MRPL46,MRPL52,MRPS10,MRPS18B,MRTO4,MSANTD1,MSL2,MSMO1,MST1R,MTA3,MTUS1,MTX2,MUC20,MVD,MVK,MYH14,MYL7,NAA15,NAA25,NAAA,NADK,NARS2,NAT10,NBAS,NCKAP1,NCSTN,NDFIP2,NDUFA11,NECTIN4,NELFE,NEPRO,NEU1,NHP2,NIFK,NIP7,NIPA2,NIPAL2,NIPSNAP3A,NKRF,NMD3,NME1,NOL11,NOL4L,NOL9,NOMO1 (includes others),NOP16,NOP2,NOP56,NOP58,NPSR1,NRTN,NSUN2,NTN1,NUCB1,NUDCD1,NUDCD2,NUMB,NUP50,NUP58,NUS1,OARD1,OSBPL3,OSBPL7,OSTC,PA2G4,PAG1,PAK1IP1,PAK2,PANK3,PAPLN,PAXBP1,PCLO,PCOLCE2,PCYT2,PDCD6,PDCD6IP,PDE12,PDZK1,PEF1,PEMT,PEPD,PES1,PEX2,PGAM5,PGLYRP1,PHF14,PHLDA1,PIGU,PIM1,PIP4K2C,PLEKHA7,PLEKHB2,PLEKHG3,PLEKHM1,PLET1,PLPP2,PLPP5,PLSCR1,PMEL,PMM2,PMVK,PNO1,PNPO,POLR2F,POLR3D,POLR3E,PON3,PPA1,PPFIA1,PPIP5K2,PPM1G,PPP1R10,PPP1R11,PPP1R14B,PPP1R15B,PPP2R1B,PPP2R2C,PPRC1,PREP,PRKAB1,PRKCE,PRMT7,PROM1,PRPF31,

Table 2D (cont'd)

		PRPF40A,PRR15,PSMA6,PSMB2,PSMB5,PSMD1,PSME3,PTBP3,PTK2,PTPRJ,PUM3,PUS1,PWWP2A,PYCR2,QRSL1,RAB11FIP1,RAB27B,RAB5IF,RAB6B,RAD23B,RALA,RALBP1,RAN,RANGAP1,RARS2,RASD1,RASGRP1,RBBP8,RBIS,RBM3,RBM34,RBM45,RBM47,RBP2,RCC1,REEP3,REL,RELCH,RER1,RET,RGP1,RIN1,RIOX2,RNF149,RNF17,RNF19A,RNF224,RNF7,RPF1,RPF2,RPH3AL,RPL7L1,RPN1,RRAS2,RRP12,RRP15,RRS1,RSPH3,RWDD4,RYBP,SC5D,SCAMP2,SCFD1,SCFD2,SCO1,SCRIB,SCTR,SDAD1,SDCBP,SEC11C,SEC13,SEC24C,SEC61A1,SEC61B,SECISBP2,SEL1L3,SELENOI,SEPHS2,SERBP1,SERP1,SETX,SF3A3,SFXN1,SH3BGRL2,SHISA2,SHOC2,SLC10A3,SLC10A7,SLC16A1,SLC16A6,SLC1A5,SLC20A1,SLC25A24,SLC30A7,SLC31A1,SLC33A1,SLC34A2,SLC34A3,SLC35A3,SLC35C1,SLC39A11,SLC39A9,SLC52A2,SLC7A1,SLC7A6OS,SLC7A7,SLCO2A1,SLCO4A1,SLCO5A1,SLITRK4,SLK,SMPDL3A,SMU1,SNAPC1,SNAPC2,SNRNP40,SNRPD1,SNU13,SNX7,SOWAHC,SOX9,SPCS2,SPECC1,SPOCK1,SPRTN,SPTLC1,SPTLC2,SPTY2D1,SQLE,SREBF2,SRM,SRP19,SRP9,SRPRB,SRSF2,SSB,SSH3,SSR1,SSR2,SSR3,SSU72,SSX2IP,STAP2,STARD4,STAT5A,STC2,STEAP1,STEAP2,STIM2,STRAP,STRIP1,STT3A,STT3B,STX19,SULF2,SWSAP1,SYPL1,TAF5L,TAPT1,TAX1BP3,TBL3,TBRG4,TCERG1,TDG,TENT5B,TEX2,TGM2,THG1L,THSD4,THYN1,TIAM1,TICAM1TINAGL1,TIPARP,TLCD3A,TLNRD1,TM9SF2,TM9SF3,TMED10,TMED2,TMED7,TMED9,TMEM128,TMEM14C,TMEM158,TMEM164,TMEM183A,TMEM190,TMEM238,TMEM248,TMEM30B,TMEM41A,TMEM41B,TMEM63A,TMEM68,TMEM87B,TNFAIP8L1,TNS4,TOMM40,TOMM70,TOR2A,TPD52,TPD52L1,TRABD,TRAF4,TRIB2,TRIM2,TRIP11,TRMT61A,TRPC3,TSFM,TSG101,TSR1,TTC13,TTC27,TTC39C,TUBA1C,TUBB4B,TWF1,TXNDC17,TXNDC9,TXNRD1,UAP1,UBA5,UBA6,UBQLN1,UBXN2A,UBXN2B,UCHL3,UCK2,UFL1,UNC13B,URB2,USO1,USP16,USP4,UTP14A,UTP14C,UTP18,UTP20,UXS1,VDAC2,VOPP1,VPS53,WAPL,WDR1,WDR36,WDR43,WDR75,WFS1,WIPI1,WWP2,XPNPEP1,XPOT,YIPF2,YIPF6,YKT6,YRDC,YWHAQ,ZBTB42,ZCCHC9,ZDHHC21,ZDHHC5,ZDHHC9,ZFP92,ZNF672,ZNF800

Table 2D (cont'd)

Cancer, Organismal Injury and Abnormalities	Solid tumor	A4GALT,AACS,ABCD3,ABCE1,ABHD11,ABHD13,ABHD6,ABI1,ACAT2,ACOT12,ACP1,ACSL3,ACTG1,ACTL6A,ACTR2,ACVR1B,ADAM10,ADAMTS3,ADGB,AGPAT2,AK2,ALAS1,ALDH18A1,ALG14,ALG3,ALG8,ALG9,AMD1,ANKRD13A,ANKRD46,AP1G1,AP1S1,AP1S2,AP2M1,AP3M1,APOBEC3B,AQP3,AQR,ARAP2,ARCN1,ARF1,ARF4,ARF6,ARFGEF1,ARFGEF2,ARHGAP12,ARHGAP32,ARHGAP39,ARPC1A,ARPC5,ARRDC4,ASAH1,ASIC2,ATAD3A,ATIC,ATP1B3,ATP4A,ATP6V0A2,ATP6V1A,AZIN1,B3GALT6,B4GALT3,BACE2,BAIAP2L1,BAK1,BARX2,BAZ1A,BBLN,BGLAP,BLCAP,BOP1,BRIX1,BTBD10,BTBD9,BUD23,BYSL,BZW1,BZW2,C11orf52,C1GALT1,C1orf226,C2CD2L,C3,C9orf64,CA5B,CAB39,CACNA2D3,CALML3,CAPN7,CAPSL,CBX4,CCDC116,CCDC25,CCDC42,CCDC65,CCDC86,CCDC88C,CCT3,CCT6A,CD2AP,CD55,CDA,CDC42SE1,CDIPT,CDV3,CEACAM19,CFAP221,CFAP52,CFB,CHD9,CHFR,CHML,CHMP2B,CHMP3,CHMP4B,CHST11,CHUK,CIAPIN1,CIPC,CISH,CLCN3,CLIC1,CLINT1,CLPTM1L,CLTC,CMPK1,CMTM6,CNBD2,COG3,COMMD10,COMMD7,COPB1,COPB2,COPZ1,CPN1,CPSF2,CRK,CRLF1,CRLS1,CROCC,CTBS,CTNNAL1,CTNND1,CTSV,CTU2,CXADR,CYB561,CYB561D2,CYP2J2,CYP51A1,CYSRT1,DAD1,DBNL,DCAF13,DCLRE1B,DDX1,DDX10,DDX21,DDX31,DDX39A,DDX3X,DDX54,DDX56,DENND2C,DENND2D,DENR,DHCR24,DHCR7,DHX32,DNAJC11,DNAJC17,DNAJC2,DNAJC3,DNTTIP2,DOK4,DOLPP1,DPAGT1,DPP3,DPY19L1,DR1,DSEL,ECE1,EDEM3,EEA1,EEF1E1,EFL1,EIF1AX,EIF2B5,EIF2S1 EIF2S2,EIF3B,EIF3D,EIF4A1,EIF4A3,EIF4E,EIF4G1,EIF5A,EIF5B,EIF6,ELOVL1,ELOVL6,ENDOD1,ENOPH1,ENTPD6,EPB41L4B,EPRS1,EPS8,ERAP1,ERG28,ERH,ERLIN2,ERO1A,ESCO1,ETF1,ETFDH,EXOC8,EXOSC1,EZR,F11R,F2RL1,F8A1 (include others), FAM136A, FAM3C,FANK1, FAR1,FARSA,FARSB,FBXO28,FDFT1,FDPS,FGFRL1,FLNB,FOXN2,FPGS,FPGT,FRG1,FRRS1,FSCN2,FUCA1,FURIN,FZD5,G3BP1,G6PD,GAK,GAL,GALE,GALNT4,GALNT7,GARS1,GCH1,GCK,GDPD1,GFER,GFPT1,GFRA4,GFUS,GIPC1,GMDS,GMPPB,GNG12,GNG4,GOLGA1,GOLGA7,GORASP2,GPATCH4,GPD1L,GPD2,GPR151,GPS1,GPX1,GRPEL2

Table 2D (cont'd)

		,GRWD1,GSPT1,GSS,GSTO1,GTF2F2,GTF3C6,GTPBP4,H2AZ1,HCRTR1,HDAC3,HEATR1,HEATR5A,HEBP2,HESX1,HGH1,HIF1A,HIP1R,HM13,HMGCR,HMGCS1,HNRNPAB,HNRNPD,HNRNPF,HORMAD2,HSD17B11,HSD17B12,HSD17B7,HSPA4,HYOU1,IBTK,IDI1,IER3,IFT43,IL17B,IL1A,IL1R1,IL24,IL36A,IL36B,INSIG1,IPO5,IQGAP2,ISYNA1,ITGA6,ITPA,JAGN1,JPT1,JPT2,JUP,KAT14,KAZN,KBTBD8,KCNF1,KCNK5,KIAA1217,KIAA2013,KIF16B,KIF21A,KIF5B,KIF5C,KLF3,KLF4,KLK11,KLK3,KPNB1,KREMEN2,KRT4,KRTCAP3,KYAT1,L2HGDH,LAMB3,LARP1,LARP4,LARS1,LDLR,LENG1,LIF,LIN7C,LIPH,LIPK,LNX2,LONRF3,LPCAT4,LPO,LRBA,LRG1,LRP8,LRRC41,LRRC59,LRRC71,LRRC8E,LSR,LSS,LTF,LTV1,LURAP1L,Ly6a (includes others),LY6D,LYAR,M6PR,MACIR,MAGOH,MAK16,MAN1A2,MANBA,MANSC1,MAP2K1,MAP2K4,MAP3K1,MAP6,MAPK6,MAPKAPK3,MAPRE1,MAT2A,MATCAP2,MBOAT2,MBTPS2,MDFI,MEA1,MED9,MET,METAP1,METTL1,MFSD2A,MFSD6,MGAT2,MICALL2,MID2,MIF4GD,MLX,MOGAT1,MORF4L2,MPDU1,MPHOSPH10,MPHOSPH6,MPZL3,MRPL17,MRPL19,MRPL20,MRPL36,MRPL46,MRPL52,MRPS10,MRPS18B,MRTO4,MSANTD1,MSL2,MSMO1,MST1R,MTA3,MTUS1,MTX2,MUC20,MVD,MVK,MYH14,MYL7,NAA15,NAA25,NAAA,NADK,NARS1,NARS2,NAT10,NBAS,NCKAP1,NCSTN,NDFIP2,NDUFA11,NECTIN4,NELFE,NEPRO,NEU1,NHP2,NIFK,NIP7,NIPA2,NIPAL2,NIPSNAP3A,NKRF,NMD3,NME1,NOL11,NOL4L,NOL9,NOMO1 (includes others),NOP16,NOP2,NOP56,NOP58,NPSR1,NRTN,NSUN2,NTN1,NUCB1,NUDCD1,NUDCD2,NUMB,NUP50,NUP58,NUS1,OARD1,OSBPL3,OSBPL7,OSTC,PA2G4,PAG1,PAK1IP1,PAK2,PANK3,PAPLN,PAXBP1,PCLO,PCOLCE2,PCYT2,XPOT,YIPF2,YIPF6,YKT6,YRDC,YWHAQ,ZBTB42,ZCCHC9,ZDHHC21,ZDHHC5,ZDHHC9,ZFP92,ZNF672,ZNF800PEX2,PGAM5,PGLYRP1,PGPEP1,PHF14,PHLDA1,PIGUPIM1,PIP4K2C,PLEKHA7,PLEKHB2,PLEKHG3,PLEKHM1,PLET1,PLPP2,PLPP5,PLSCR1,PMEL,PMM2,PMVK,PNO1,PNPO,POLR2F,POLR3D,POLR3E

Table 2D (cont'd)

		,PON3,PPA1,PPFIA1,PPIP5K2,PPM1G,PPP1R10,PPP1R11,PPP1R14B,PPP1R15B,PPP2R1B,PPP2R2C,PPRC1,PREP,PRKAB1,PRKCE,PRMT7,PROM1,PRPF31,PRPF40A,PRR15,PSMA6,PSMB2,PSMB5,PSMD1,PSME3,PTBP3,PTK2,PTPRJ,PUM3,PUS1,PWWP2A,PYCR2,QRSL1,RAB11FIP1,RAB27B,RAB5IF,RAB6B,RAD23B,RALA,RALBP1,RAN,RANGAP1,RARS2,RASD1,RASGRP1,RBBP8,RBIS,RBM3,RBM34,RBM45,RBM47,RBP2,RCC1,REEP3,REL,RELCH,RER1,RET,RGP1,RIN1,RIOX2,RNF149,RNF17,RNF19A,RNF224,RNF7,RPF1,RPF2,RPH3AL,RPL7L1,RPN1,RRAS2,Rrbp1,RRP12,RRP15,RRS1,RSPH3,RWDD4,RYBP,SC5D,SCAMP2,SCFD1,SCFD2,SCO1,SCRIB,SCTR,SDAD1,SDCBP,SEC11C,SEC13,SEC24C,SEC61A1,SEC61B,SECISBP2,SEL1L3,SELENOI,SEPHS2,SERBP1,SERP1,SETX,SF3A3,SFXN1,SH3BGRL2,SHISA2,SHOC2,SKIC3,SLC10A3,SLC10A7,SLC16A1,SLC16A6,SLC1A5,SLC20A1,SLC25A24,SLC30A7,SLC31A1,SLC33A1,SLC34A2,SLC34A3,SLC35A3,SLC35C1,SLC39A11,SLC39A9,SLC52A2,SLC7A1,SLC7A6OS,SLC7A7,SLCO2A1,SLCO4A1,SLCO5A1,SLITRK4,SLK,SMPDL3A,SMU1,SNAPC1,SNAPC2,SNRNP40,SNRPD1,SNU13,SNX7,SOWAHC,SOX9,SPCS2,SPECC1,SPOCK1,SPRTN,SPTLC1,SPTLC2,SPTY2D1,SQLE,SREBF2,SRM,SRP19,SRP9,SRPRB,SRSF2,SSB,SSH3,SSR1,SSR2,SSR3,SSU72,SSX2IP,STAP2,STARD4,STAT5A,STC2,STEAP1,STEAP2,STIM2,STRAP,STRIP1,STT3A,STT3B,STX19,SULF2,SWSAP1,SYPL1,TAF5L,TAPT1,TAX1BP3,TBL3,TBRG4,TCERG1,TDG,TENT5B,TEX2,TGM2,THG1L,THSD4,THYN1,TIAM1,TICAM1,TINAGL1,TIPARP,TLCD3A,TLNRD1,TM9SF2,TM9SF3,TMED10,TMED2,TMED7,TMED9,TMEM128,TMEM14C,TMEM158,TMEM164,TMEM183A,TMEM190,TMEM238,TMEM248,TMEM30B,TMEM41A,TMEM41B,TMEM63A,TMEM68,TMEM87B,Tmsb4x (includes others), TNFAIP8L1,TNS4,TOMM40,TOMM70,TOR2A,TPD52,TPD52L1,TRABD,TRAF4,TRIB2,TRIM2,TRIP11,TRMT61A,TRPC3,TSFM,TSG101,TSR1,TTC13,TTC27,TTC39C,TUBA1C,TUBB4B,TWF1,TXNDC17,TXNDC9,TXNRD1,UAP1,UBA5,UBA6,UBQLN1,UBXN2A,UBXN2B,UCHL3,UCK2,UFL1,UNC13B,URB2,US

Table 2D (cont'd)

		O1,USP16,USP4,UTP14A,UTP14C,UTP18,UTP20, UXS1,VDAC2,VOPP1,VPS53,WAPL,WDR1,WDR36 ,WDR43,WDR75,WFS1,WIPI1,WWP2,XPNPEP1,
Cancer, Organismal Injury and Abnormalities	Cancer	A4GALT,AACS,ABCD3,ABCE1,ABHD11,ABHD13,A BHD6,ABI1,ACAT2,ACOT12,ACP1,ACSL3,ACTG1, ACTL6A,ACTR2,ACVR1B,ADAM10,ADAMTS3,ADG B,AGPAT2,AK2,ALAS1,ALDH18A1,ALG14,ALG3,AL G8,ALG9,AMD1,ANKRD13A,ANKRD46,AP1G1,AP1 S1,AP1S2,AP2M1,AP3M1,APOBEC3B,AQP3,AQR, ARAP2,ARCN1,ARF1,ARF4,ARF6,ARFGEF1,ARFG EF2,ARHGAP12,ARHGAP32,ARHGAP39,ARPC1A, ARPC5,ARRDC4,ASAH1,ASIC2,ATAD3A,ATIC,ATP 1B3,ATP4A,ATP6V0A2,ATP6V1A,AZIN1,B3GALT6, B4GALT3,BACE2,BAIAP2L1,BAK1,BARX2,BAZ1A, BBLN,BGLAP,BLCAP,BOP1,BRIX1,BTBD10,BTBD9 ,BUD23,BYSL,BZW1,BZW2,C11orf52,C1GALT1,C1 orf226,C2CD2L,C3,C9orf64,CA5B,CAB39,CACNA2 D3,CALML3,CAPN7,CAPSL,CBX4,CCDC116,CCD C25,CCDC42,CCDC65,CCDC86,CCDC88C,CCT3, CCT6A,CD2AP,CD55,CDA,CDC42SE1,CDIPT,CDV 3,CEACAM19,CFAP221,CFAP52,CFB,CHD9,CHFR ,CHML,CHMP2B,CHMP3,CHMP4B,CHST11,CHUK, CIAPIN1,CIPC,CISH,CLCN3,CLIC1,CLINT1,CLPTM 1L,CLTC,CMPK1,CMTM6,CNBD2,COG3,COMMD1 0,COMMD7,COPB1,COPB2,COPZ1,CPN1,CPSF2, CRK,CRLF1,CRLS1,CROCC,CTBS,CTNNAL1,CTN ND1,CTSV,CTU2,CXADR,CYB561,CYB561D2,CYP 2J2,CYP51A1,CYSRT1,DAD1,DBNL,DCAF13,DCL RE1B,DDX1,DDX10,DDX21,DDX31,DDX39A,DDX3 X,DDX54,DDX56,DENND2C,DENND2D,DENR,DHC R24,DHCR7,DHX32,DNAJC11,DNAJC17,DNAJC2, DNAJC3,DNTTIP2,DOK4,DOLPP1,DPAGT1,DPP3, DPY19L1,DSEL,ECE1,EDEM3,EEA1,EEF1E1,EFL1 ,EIF1AX,EIF2B5,EIF2S1,EIF2S2,EIF3B,EIF3D,EIF4 A1,EIF4A3,EIF4E,EIF4G1,EIF5A,EIF5B,EIF6,ELOV L1,ELOVL6,ENDOD1,ENOPH1,ENTPD6,EPB41L4B ,EPRS1,EPS8,ERAP1,ERG28,ERH,ERLIN2,ERO1A ,ESCO1,ETF1,ETFDH,EXOC8,EXOSC1,EZR,F11R, F2RL1,F8A1 (includes others),FAM136A, FAM3C,FANK1,FAR1,FARSA,FARSB,FBXO28,FDF T1,FDPS,FGFRL1,FLNB,FOXN2,FPGS,FPGT,FRG 1,FRRS1,FSCN2,FUCA1,FURIN,FZD5,G3BP1,G6P D,GAK,GAL,GALE,GALNT4,GALNT7,GARS1,GCH1 ,GCK,GDPD1,GFER,GFPT1,GFRA4,GFUS,GIPC1,

Table 2D (cont'd)

		GMDS,GMPPB,GNG12,GNG4,GOLGA1,GOLGA7, GORASP2,GPATCH4,GPD1L,GPD2,GPR151,GPS1 ,GPX1,GRPEL2,GRWD1,GSPT1,GSS,GSTO1,GTF 2F2,GTF3C6,GTPBP4,H2AZ1,HCRTR1,HDAC3,HE ATR1,HEATR5A,HEBP2,HESX1,HGH1,HIF1A,HIP1 R,HM13,HMGCR,HMGCS1,HNRNPAB,HNRNPD,H NRNPF,HORMAD2,HSD17B11,HSD17B12,HSD17B 7,HSPA4,HYOU1,IBTK,IDI1,IER3,IFT43,IL17B,IL1A, IL1R1,IL24,IL36A,IL36B,INSIG1,IPO5,IQGAP2,ISYN A1,ITGA6,ITPA,JAGN1,JPT1,JPT2,JUP,KAT14,KAZ N,KBTBD8,KCNF1,KCNK5,KIAA1217,KIAA2013,KIF 16B,KIF21A,KIF5B,KIF5C,KLF3,KLF4,KLK11,KLK3, KPNB1,KREMEN2,KRT4,KRTCAP3,KYAT1,L2HGD H,LAMB3,LARP1,LARP4,LARS1,LDLR,LENG1,LIF, LIN7C,LIPH,LIPK,LNX2,LONRF3,LPCAT4,LPO,LRB A,LRG1,LRP8,LRRC41,LRRC59,LRRC71,LRRC8E, LSR,LSS,LTF,LTV1,LURAP1L,Ly6a (includes others),LY6D,LYAR,M6PR,MACIR,MAGOH,MAK16, MAN1A2,MANBA,MANSC1,MAP2K1,MAP2K4,MAP 3K1,MAP6,MAPK6,MAPKAPK3,MAPRE1,MAT2A,M ATCAP2,MBOAT2,MBTPS2,MDFI,MEA1,MED9,ME T,METAP1,METTL1,MFSD2A,MFSD6,MGAT2,MIC ALL2,MID2,MIF4GD,MLX,MOGAT1,MORF4L2,MPD U1,MPHOSPH10,MPHOSPH6,MPZL3,MRPL17,MR PL19,MRPL20,MRPL36,MRPL46,MRPL52,MRPS10 ,MRPS18B,MRTO4,MSANTD1,MSL2,MSMO1,MST 1R,MTA3,MTUS1,MTX2,MUC20,MVD,MVK,MYH14, MYL7,NAA15,NAA25,NAAA,NADK,NARS1,NARS2, NAT10,NBAS,NCKAP1,NCSTN,NDFIP2,NDUFA11, NECTIN4,NELFE,NEPRO,NEU1,NHP2,NIFK,NIP7, NIPA2,NIPAL2,NIPSNAP3A,NKRF,NMD3,NME1,NO L11,NOL4L,NOL9,NOMO1 (includes others), NOP16,NOP2,NOP56,NOP58,NPSR1,NRTN,NSUN 2,NTN1,NUCB1,NUDCD1,NUDCD2,NUMB,NUP50, NUP58,NUS1,OARD1,OSBPL3,OSBPL7,OSTC,PA2 G4,PAG1,PAK1IP1,PAK2,PANK3,PAPLN,PAXBP1, PCLO,PCOLCE2,PCYT2,PDCD6,PDCD6IP,PDE12, PDZK1,PEF1,PEMT,PEPD,PES1,PEX2,PGAM5,PG LYRP1,PGPEP1,PHF14,PHLDA1,PIGU,PIM1,PIP4K 2C,PLEKHA7,PLEKHB2,PLEKHG3,PLEKHM1,PLET 1,PLPP2,PLPP5,PLSCR1,PMEL,PMM2,PMVK,PNO 1,PNPO,POLR2F,POLR3D,POLR3E,PON3,PPA1,P PFIA1,PPIP5K2,PPM1G,PPP1R10,PPP1R11,PPP1 R14B,PPP1R15B,PPP2R1B,PPP2R2C,PPRC1,

Table 2D (cont'd)

		PREP,PRKAB1,PRKCE,PRMT7,PROM1,PRPF31,PRPF40A,PRR15,PSMA6,PSMB2,PSMB5,PSMD1,PSME3,PTBP3,PTK2,PTPRJ,PUM3,PUS1,PWWP2A,PYCR2,QRSL1,RAB11FIP1,RAB27B,RAB5IF,RAB6B,RAD23B,RALA,RALBP1,RAN,RANGAP1,RARS2,RASD1,RASGRP1,RBBP8,RBIS,RBM3,RBM34,RBM45,RBM47,RBP2,RCC1,REEP3,REL,RELCH,RER1,RET,RGP1,RIN1,RIOX2,RNF149,RNF17,RNF19A,RNF224,RNF7,RPF1,RPF2,RPH3AL,RPL7L1,RPN1,RRAS2,Rrbp1,RRP12,RRP15,RRS1,RSPH3,RWDD4,RYBP,SC5D,SCAMP2,SCFD1,SCFD2,SCO1,SCRIB,SCTR,SDAD1,SDCBP,SEC11C,SEC13,SEC24C,SEC61A1,SEC61B,SECISBP2,SEL1L3,SELENOI,SEPHS2,SERBP1,SERP1,SETX,SF3A3,SFXN1,SH3BGRL2,SHISA2,SHOC2,SKIC3,SLC10A3,SLC10A7,SLC16A1,SLC16A6,SLC1A5,SLC20A1,SLC25A24,SLC30A7,SLC31A1,SLC33A1,SLC34A2,SLC34A3,SLC35A3,SLC35C1,SLC39A11,SLC39A9,SLC52A2,SLC7A1,SLC7A6OS,SLC7A7,SLCO2A1,SLCO4A1,SLCO5A1,SLITRK4,SLK,SMPDL3A,SMU1,SNAPC1,SNAPC2,SNRNP40,SNRPD1,SNU13,SNX7,SOWAHC,SOX9,SPCS2,SPECC1,SPOCK1,SPRTN,SPTLC1,SPTLC2,SPTY2D1,SQLE,SREBF2,SRM,SRP19,SRP9,SRPRB,SRSF2,SSB,SSH3,SSR1,SSR2,SSR3,SSU72,SSX2IP,STAP2,STARD4,STAT5A,STC2,STEAP1,STEAP2,STIM2,STRAP,STRIP1,STT3A,STT3B,STX19,SULF2,SWSAP1,SYPL1,TAF5L,TAPT1,TAX1BP3,TBL3,TBRG4,TCERG1,TDG,TENT5B,TEX2,TGM2,THG1L,THSD4,THYN1,TIAM1,TICAM1,TINAGL1,TIPARP,TLCD3A,TLNRD1,TM9SF2,TM9SF3,TMED10,TMED2,TMED7,TMED9,TMEM128,TMEM14C,TMEM158,TMEM164,TMEM183A,TMEM190,TMEM238,TMEM248,TMEM30B,TMEM41A,TMEM41B,TMEM63A,TMEM68,TMEM87B,TNFAIP8L1,TNS4,TOMM40,TOMM70,TOR2A,TPD52,TPD52L1,TRABD,TRAF4,TRIB2,TRIM2,TRIP11,TRMT61A,TRPC3,TSFM,TSG101,TSR1,TTC13,TTC27,TTC39C,TUBA1C,TUBB4B,TVP23B,TWF1,TXNDC17,TXNDC9,TXNRD1,U2af1,UAP1,UBA5,UBA6,UBQLN1,UBXN2A,UBXN2B,UCHL3,UCK2,UFL1,UNC13B,URB2,USO1,USP16,USP4,UTP14A,UTP14C,UTP18,UTP20,UXS1,VDAC2,VOPP1,VPS53,WAPL,WDR1,WDR36,WDR43,WDR75,WFS1,WIPI1,WWP2,XPNPEP1,XPOT,YIPF2,YIPF6,YKT6,YRDC,YWHAQ,ZBTB42,

Table 2D (cont'd)

		ZCCHC9,ZDHHC21,ZDHHC5,ZDHHC9,ZFP92,ZNF672,ZNF800
Cancer, Organismal Injury and Abnormalities	Extracranial solid tumor	A4GALT,AACS,ABCD3,ABCE1,ABHD11,ABHD13,ABHD6,ABI1,ACAT2,ACOT12,ACP1,ACSL3,ACTG1,ACTL6A,ACTR2,ACVR1B,ADAM10,ADAMTS3,ADGB,AGPAT2,AK2,ALAS1,ALDH18A1,ALG14,ALG3,ALG8,ALG9,AMD1,ANKRD13A,ANKRD46,AP1G1,AP1S1,AP1S2,AP2M1,AP3M1,APOBEC3B,AQP3,AQR,ARAP2,ARCN1,ARF1,ARF4,ARF6,ARFGEF1,ARFGEF2,ARHGAP12,ARHGAP32,ARHGAP39,ARPC1A,ARPC5,ARRDC4,ASAH1,ASIC2,ATAD3A,ATIC,ATP1B3,ATP4A,ATP6V0A2,ATP6V1A,AZIN1,B3GALT6,B4GALT3,BACE2,BAIAP2L1,BAK1,BARX2,BAZ1A,BBLN,BGLAP,BLCAP,BOP1,BRIX1,BTBD10,BTBD9,BUD23,BYSL,BZW1,BZW2,C11orf52,C1GALT1,C1orf226,C2CD2L,C3,C9orf64,CA5B,CAB39,CACNA2D3,CALML3,CAPN7,CAPSL,CBX4,CCDC116,CCDC25,CCDC42,CCDC65,CCDC86,CCDC88C,CCT3,CCT6A,CD2AP,CD55,CDA,CDC42SE1,CDIPT,CDV3,CEACAM19,CFAP221,CFAP52,CFB,CHD9,CHFR,CHML,CHMP2B,CHMP3,CHMP4B,CHST11,CHUK,CIAPIN1,CIPC,CISH,CLCN3,CLIC1,CLINT1,CLPTM1L,CLTC,CMPK1,CMTM6,CNBD2,COG3,COMMD10,COMMD7,COPB1,COPB2,COPZ1,CPN1,CPSF2,CRK,CRLF1,CRLS1,CROCC,CTBS,CTNNAL1,CTNND1,CTSV,CTU2,CXADR,CYB561,CYB561D2,CYP2J2,CYP51A1,CYSRT1,DAD1,DBNL,DCAF13,DCLRE1B,DDX1,DDX10,DDX21,DDX31,DDX39A,DDX3X,DDX54,DDX56,DENND2C,DENND2D,DENR,DHCR24,DHCR7,DHX32,DNAJC11,DNAJC17,DNAJC2,DNAJC3,DNTTIP2,DOK4,DOLPP1,DPAGT1,DPP3,DPY19L1,DR1,DSEL,ECE1,EDEM3,EEA1,EEF1E1,EFL1,EIF1AX,EIF2B5,EIF2S1,EIF2S2,EIF3B,EIF3D,EIF4A1,EIF4A3,EIF4E,EIF4G1,EIF5A,EIF5B,EIF6,ELOVL1,ELOVL6,ENDOD1,ENOPH1,ENTPD6,EPB41L4B,EPS8,ERAP1,ERG28,ERH,ERLIN2,ERO1A,ESCO1,ETF1,ETFDH,EXOC8,EXOSC1,EZR,F11R,F2RL1,F8A1 (includes others),FAM136A,FAM3C,FANK1,FAR1,FARSA,FARSB,FBXO28,FDFT1,FDPS,FGFRL1,FLNB,FOXN2,FPGS,FPGT,FRG1,FRRS1,FSCN2,FUCA1,FURIN,FZD5,G3BP1,G6PD,GAK,GAL,GALE,GALNT4,GALNT7

Table 2D (cont'd)

		,GARS1,GCH1,GCK,GDPD1,GFER,GFPT1,GFRA4,GFUS,GIPC1,GMDS,GMPPB,GNG12,GNG4,GOLGA1,GOLGA7,GORASP2,GPATCH4,GPD1L,GPD2,GPR151,GPS1,GPX1,GRPEL2,GRWD1,GSPT1,GSS,GSTO1,GTF2F2,GTF3C6,GTPBP4,HCRTR1,HDAC3,HEATR1,HEATR5A,HEBP2,HESX1,HGH1,HIF1A,HIP1R,HM13,HMGCR,HMGCS1,HNRNPAB,HNRNPD,HNRNPF,HORMAD2,HSD17B11,HSD17B12,HSD17B7,HSPA4,HYOU1,IBTK,IDI1,IER3,IFT43,IL17B,IL1A,IL1R1,IL24,IL36A,IL36B,INSIG1,IPO5,IQGAP2,ISYNA1,ITGA6,ITPA,JAGN1,JPT1,JPT2,JUP,KAT14,KAZN,KBTBD8,KCNF1,KCNK5,KIAA1217,KIAA2013,KIF16B,KIF21A,KIF5B,KIF5C,KLF3,KLF4,KLK11,KLK3,KPNB1,KREMEN2,KRT4,KRTCAP3,KYAT1,L2HGDH,LAMB3,LARP1,LARP4,LARS1,LDLR,LENG1,LIF,LIN7C,LIPH,LIPK,LNX2,LONRF3,LPCAT4,LPO,LRBA,LRG1,LRP8,LRRC41,LRRC59,LRRC71,LRRC8E,LSR,LSS,LTF,LTV1,LURAP1L,Ly6a (includes others),LY6D,LYAR,M6PR,MACIR,MAGOH,MAK16,MAN1A2,MANBA,MANSC1,MAP2K1,MAP2K4,MAP3K1,MAP6,MAPK6,MAPKAPK3,MAPRE1,MAT2A,MATCAP2,MBOAT2, MBTPS2,MDFI,MEA1,MED9,MET,METAP1,METTL1,MFSD2A,MFSD6,MGAT2,MICALL2,MID2,MIF4GD,MLX,MOGAT1,MORF4L2,MPDU1,MPHOSPH10,MPHOSPH6,MPZL3,MRPL17,MRPL19,MRPL20,MRPL36,MRPL46,MRPL52,MRPS10,MRPS18B,MRTO4,MSANTD1,MSL2,MSMO1,MST1R,MTA3,MTUS1,MTX2,MUC20,MVD,MVK,MYH14,MYL7,NAA15,NAA25,NAAA,NADK,NARS2,NAT10,NBAS,NCKAP1,NCSTN,NDFIP2,NDUFA11,NECTIN4,NELFE,NEPRO,NEU1,NHP2,NIFK,NIP7,NIPA2,NIPAL2,NIPSNAP3A,NKRF,NMD3,NME1,NOL11,NOL4L,NOL9,NOMO1 (includes others),NOP16,NOP2,NOP56,NOP58,NPSR1,NRTN,NSUN2,NTN1,NUCB1,NUDCD1,NUDCD2,NUMB,NUP50,NUP58,NUS1,OARD1,OSBPL3,OSBPL7,OSTC,PA2G4,PAG1,PAK1IP1,PAK2,PANK3,PAPLN,PAXBP1,PCLO,PCOLCE2,PCYT2,PDCD6,PDCD6IP,PDE12,PDZK1,PEF1,PEMT,PEPD,PES1,PEX2,PGAM5,PGLYRP1,PGPEP1,PHF14,PHLDA1,PIGU,PIM1,PIP4K2C,PLEKHA7,PLEKHB2,PLEKHG3,PLEKHM1,PLET1,PLPP2,PLPP5,PLSCR1,PMEL,PMM2,PMVK

Table 2D (cont'd)

		,PNO1,PNPO,POLR2F,POLR3D,POLR3E,PON3,PPA1,PPFIA1,PPIP5K2,PPM1G,PPP1R10,PPP1R11,PPP1R14B,PPP1R15B,PPP2R1B,PPP2R2C,PPRC1,PREP,PRKAB1,PRKCE,PRMT7,PROM1,PRPF31,PRPF40A,PRR15,PSMA6,PSMB2,PSMB5,PSMD1,PSME3,PTBP3,PTK2,PTPRJ,PUM3,PUS1,PWWP2A,PYCR2,QRSL1,RAB11FIP1,RAB27B,RAB5IF,RAB6B,RAD23B,RALA,RALBP1,RAN,RANGAP1,RARS2,RASD1,RASGRP1,RBBP8,RBIS,RBM3,RBM34,RBM45,RBM47,RBP2,RCC1,REEP3,REL,RELCH,RER1,RET,RGP1,RIN1,RIOX2,RNF149,RNF17,RNF19A,RNF224,RNF7,RPF1,RPF2,RPH3AL,RPL7L1,RPN1,RRAS2,Rrbp1,RRP12,RRP15,RRS1,RSPH3,RWDD4,RYBP,SC5D,SCAMP2,SCFD1,SCFD2,SCO1,SCRIB,SCTR,SDAD1,SDCBP,SEC11C,SEC13,SEC24C,SEC61A1,SEC61B,SECISBP2,SEL1L3,SELENOI,SEPHS2,SERBP1,SERP1,SETX,SF3A3,SFXN1,SH3BGRL2,SHISA2,SHOC2,SKIC3,SLC10A3,SLC10A7,SLC16A1,SLC16A6,SLC1A5,SLC20A1,SLC25A24,SLC30A7,SLC31A1,SLC33A1,SLC34A2,SLC34A3,SLC35A3,SLC35C1,SLC39A11,SLC39A9,SLC52A2,SLC7A1,SLC7A6OS,SLC7A7,SLCO2A1,SLCO4A1,SLCO5A1,SLITRK4,SLK,SMPDL3A,SMU1,SNAPC1,SNAPC2,SNRNP40,SNRPD1,SNU13,SNX7,SOWAHC,SOX9,SPCS2,SPECC1,SPOCK1,SPRTN,SPTLC1,SPTLC2,SPTY2D1,SQLE,SREBF2,SRM,SRP19,SRP9,SRPRB,SRSF2,SSB,SSH3,SSR1,SSR2,SSR3,SSU72,SSX2IP,STAP2,STARD4,STAT5A,STC2,STEAP1,STEAP2,STIM2,STRAP,STRIP1,STT3A,STT3B,STX19,SULF2,SWSAP1,SYPL1,TAF5L,TAPT1,TAX1BP3,TBL3,TBRG4,TCERG1,TDG,TENT5B,TEX2,TGM2,THG1L,THSD4,THYN1,TIAM1,TICAM1,TINAGL1,TIPARP,TLCD3A,TLNRD1,TM9SF2,TM9SF3,TMED10,TMED2,TMED7,TMED9,TMEM128,TMEM14C,TMEM158,TMEM164,TMEM183A,TMEM190,TMEM238,TMEM248,TMEM30B,TMEM41A,TMEM41B,TMEM63A,TMEM68,TMEM87B,Tmsb4x (includes others),TNFAIP8L1,TNS4,TOMM40,TOMM70,TOR2A,TPD52,TPD52L1,TRABD,TRAF4,TRIB2,TRIM2,TRIP11,TRMT61A,TRPC3,TSFM,TSG101,TSR1,TTC13,TTC27,TTC39C,TUBA1C,TUBB4B,TWF1,TXNDC9,TXNRD1,UAP1,UBA5,UBA6,UBQLN1,UBXN2A,

Table 2D (cont'd)

		UBXN2B,UCHL3,UCK2,UFL1,UNC13B,URB2,USO1,USP16,USP4,UTP14A,UTP14C,UTP18,UTP20,UXS1,VDAC2,VOPP1,VPS53,WAPL,WDR1,WDR36,WDR43,WDR75,WFS1,WIPI1,WWP2,XPNPEP1,XPOT,YIPF2,YIPF6,YKT6,YRDC,YWHAQ,ZBTB42,ZCCHC9,ZDHHC21,ZDHHC5,ZDHHC9,ZFP92,ZNF672,ZNF800
Cancer, Organismal Injury and Abnormalities	Non-melanoma solid tumor	AACS,ABCD3,ABCE1,ABHD11,ABHD13,ABHD6,ABI1,ACAT2,ACOT12,ACP1,ACSL3,ACTG1,ACTR2,ACVR1B,ADAM10,ADAMTS3,ADGB,AGPAT2,AK2,ALAS1,ALDH18A1,ALG14,ALG3,ALG8,ALG9,AMD1,ANKRD13A,AP1G1,AP1S1,AP1S2,AP2M1,AP3M1,APOBEC3B,AQP3,AQR,ARAP2,ARCN1,ARF1,ARF4,ARF6,ARFGEF1,ARFGEF2,ARHGAP12,ARHGAP32,ARHGAP39,ARPC1A,ARPC5,ARRDC4,ASAH1,ASIC2,ATAD3A,ATIC,ATP1B3,ATP4A,ATP6V0A2,ATP6V1A,AZIN1,B3GALT6,B4GALT3,BACE2,BAIAP2L1,BAK1,BARX2,BAZ1A,BBLN,BGLAP,BLCAP,BOP1,BRIX1,BTBD10,BTBD9,BUD23,BYSL,BZW1,BZW2,C11orf52,C1GALT1,C1orf226,C2CD2L,C3,C9orf64,CA5B,CAB39,CACNA2D3,CAPN7,CAPSL,CBX4,CCDC116,CCDC25,CCDC42,CCDC65,CCDC86,CCDC88C,CCT3,CCT6A,CD2AP,CD55,CDA,CDC42SE1,CDIPT,CDV3,CEACAM19,CFAP221,CFAP52,CFB,CHD9,CHFR,CHML,CHMP2B,CHMP3,CHMP4B,CHST11,CHUK,CIAPIN1,CIPC,CISH,CLCN3,CLIC1,CLINT1,CLPTM1L,CLTC,CMPK1,CMTM6,CNBD2,COG3,COMMD10,COMMD7,COPB1,COPB2,COPZ1,CPN1,CPSF2,CRK,CRLF1,CRLS1,CROCC,CTBS,CTNNAL1,CTNND1,CTSV,CTU2,CXADR,CYB561,CYB561D2,CYP2J2,CYP51A1,CYSRT1,DAD1,DBNL,DCAF13,DCLRE1B,DDX1,DDX10,DDX21,DDX31,DDX39A,DDX3X,DDX54,DDX56,DENND2C,DENND2D,DENR,DHCR24,DHCR7,DHX32,DNAJC11,DNAJC17,DNAJC2,DNAJC3,DNTTIP2,DOK4,DOLPP1,DPAGT1,DPP3,DPY19L1,DR1,DSEL,ECE1,EDEM3,EEA1,EEF1E1,EFL1,EIF1AX,EIF2B5,EIF2S1,EIF2S2,EIF3B,EIF3D,EIF4A1,EIF4A3,EIF4E,EIF4G1,EIF5A,EIF5B,EIF6,ELOVL1,ELOVL6,ENDOD1,ENOPH1,ENTPD6,EPB41L4B,EPRS1,EPS8,ERAP1,ERG28,ERH,ERLIN2,ERO1A,ESCO1,ETF1,ETFDH,EXOC8,EXOSC1,EZR,F11R,F2RL1,F8A1 (includes

Table 2D (cont'd)

		others),FAM136A,FAM3C,FANK1,FAR1,FARSA,FARSB,FBXO28,FDFT1,FDPS,FGFRL1,FLNB,FOXN2,FPGS,FPGT,FRG1,FRRS1,FSCN2,FUCA1,FURIN,FZD5,G3BP1,G6PD,GAK,GAL,GALE,GALNT4,GALNT7,GCH1,GCK,GDPD1,GFER,GFPT1,GFRA4,GIPC1,GMDS,GMPPB,GNG12,GNG4,GOLGA1,GOLGA7,GORASP2,GPATCH4,GPD1L,GPD2,GPR151,GPS1,GPX1,GRPEL2,GRWD1,GSPT1,GSS,GSTO1,GTF2F2,GTF3C6,GTPBP4,H2AZ1,HCRTR1,HDAC3,HEATR1,HEATR5A,HEBP2,HESX1,HGH1,HIF1A,HIP1R,HM13,HMGCR,HMGCS1,HNRNPAB,HNRNPD,HNRNPF,HORMAD2,HSD17B11,HSD17B12,HSD17B7,HSPA4,HYOU1,IBTK,IDI1,IER3,IFT43,IL17B,IL1A,IL1R1,IL24,IL36A,IL36B,INSIG1,IPO5,IQGAP2,ISYNA1,ITGA6,ITPA,JAGN1,JPT1,JPT2,JUP,KAT14,KAZN,KBTBD8,KCNF1,KCNK5,KIAA1217,KIAA2013,KIF16B,KIF21A,KIF5B,KIF5C,KLF3,KLF4,KLK11,KLK3,KPNB1,KREMEN2,KRT4,KRTCAP3,KYAT1,L2HGDH,LAMB3,LARP1,LARP4,LDLR,LENG1,LIF,LIN7C,LIPH,LIPK,LNX2,LONRF3,LPCAT4,LPO,LRBA,LRG1,LRP8,LRRC41,LRRC59,LRRC71,LRRC8E,LSR,LSS,LTF,LTV1,LURAP1L,Ly6a (includes others),LY6D,LYAR,M6PR,MACIR,MAGOH,MAK16,MAN1A2,MANBA,MANSC1,MAP2K1,MAP2K4,MAP3K1,MAP6,MAPK6,MAPKAPK3,MAPRE1,MAT2A,MATCAP2,MBOAT2,MBTPS2,MDFI,MEA1,MED9,MET,METAP1,METTL1,MFSD2A,MFSD6,MGAT2,MICALL2,MID2,MIF4GD,MLX,MOGAT1,MORF4L2,MPDU1,MPHOSPH10,MPHOSPH6,MPZL3,MRPL17,MRPL19,MRPL20,MRPL36,MRPL46,MRPL52,MRPS10,MRPS18B,MRTO4,MSANTD1,MSL2,MSMO1,MST1R,MTA3,MTUS1,MTX2,MUC20,MVD,MVK,MYH14,MYL7,NAA15,NAA25,NAAA,NADK,NARS1,NARS2,NAT10,NBAS,NCKAP1,NCSTN,NDFIP2,NDUFA11,NECTIN4,NELFE,NEPRO,NEU1,NHP2,NIFK,NIP7,NIPA2,NIPAL2,NIPSNAP3A,NKRF,NMD3,NME1,NOL11,NOL4L,NOL9,NOMO1 (includes others),NOP16,NOP2,NOP56,NOP58,NPSR1,NRTN,NSUN2,NTN1,NUCB1,NUDCD1,NUDCD2,NUMB,NUP50,NUP58,NUS1,OARD1,OSBPL3,OSBPL7,OSTC,PA2G4,PAG1,PAK1IP1,PAK2,PANK3,PAPLN,PAXBP1,PCLO,PCOLCE2,PCYT2,PDCD6,PDCD6IP,PDE12,PDZK1,PEF1,PEMT,PEPD,PES1,PEX2,PGLYRP1,PHF14,PHLDA1,PIGU,PIM1,PIP4K2C

Table 2D (cont'd)

		,PLEKHA7,PLEKHB2,PLEKHG3,PLEKHM1,PLET1, PLPP2,PLPP5,PLSCR1,PMEL,PMM2,PMVK,PNO1, PNPO,POLR2F,POLR3D,POLR3E,PON3,PPA1,PP FIA1,PPIP5K2,PPM1G,PPP1R10,PPP1R11,PPP1R 14B,PPP1R15B,PPP2R1B,PPP2R2C,PPRC1,PREP ,PRKAB1,PRKCE,PRMT7,PROM1,PRPF31,PRPF4 0A,PRR15,PSMA6,PSMB2,PSMB5,PSMD1,PSME3, PTBP3,PTK2,PTPRJ,PUM3,PUS1,PWWP2A,PYCR 2,QRSL1,RAB11FIP1,RAB27B,RAB5IF,RAB6B,RA D23B,RALA,RALBP1,RAN,RANGAP1,RARS2,RAS D1,RASGRP1,RBBP8,RBIS,RBM3,RBM34,RBM45, RBM47,RBP2,RCC1,REEP3,REL,RELCH,RER1,RE T,RGP1,RIN1,RIOX2,RNF149,RNF17,RNF19A,RNF 224,RNF7,RPF1,RPF2,RPH3AL,RPL7L1,RPN1,RR AS2,RRP12,RRP15,RRS1,RSPH3,RWDD4,RYBP,S C5D,SCAMP2,SCFD1,SCFD2,SCO1,SCRIB,SCTR, SDAD1,SDCBP,SEC11C,SEC13,SEC24C,SEC61A 1,SEC61B,SECISBP2,SEL1L3,SEPHS2,SERBP1,S ERP1,SETX,SF3A3,SFXN1,SH3BGRL2,SHISA2,SH OC2,SKIC3,SLC10A3,SLC10A7,SLC16A1,SLC16A6 ,SLC1A5,SLC20A1,SLC25A24,SLC30A7,SLC31A1, SLC33A1,SLC34A2,SLC34A3,SLC35A3,SLC35C1, SLC39A11,SLC39A9,SLC52A2,SLC7A1,SLC7A6OS ,SLC7A7,SLCO2A1,SLCO4A1,SLCO5A1,SLITRK4, SLK,SMPDL3A,SMU1,SNAPC1,SNAPC2,SNRNP40 ,SNRPD1,SNU13,SNX7,SOWAHC,SOX9,SPCS2,S PECC1,SPOCK1,SPRTN,SPTLC1,SPTLC2,SPTY2 D1,SQLE,SREBF2,SRM,SRP19,SRP9,SRPRB,SRS F2,SSB,SSH3,SSR1,SSR2,SSR3,SSU72,SSX2IP,S TAP2,STAT5A,STC2,STEAP1,STEAP2,STIM2,STR AP,STRIP1,STT3A,STT3B,STX19,SULF2,SWSAP1, SYPL1,TAF5L,TAPT1,TAX1BP3,TBL3,TBRG4,TCE RG1,TDG,TENT5B,TEX2,TGM2,THG1L,THSD4,TH YN1,TIAM1,TICAM1,TINAGL1,TIPARP,TLCD3A,TL NRD1,TM9SF2,TM9SF3,TMED10,TMED2,TMED7,T MED9,TMEM128,TMEM14C,TMEM158,TMEM164,T MEM183A,TMEM190,TMEM238,TMEM248,TMEM3 0B,TMEM41A,TMEM41B,TMEM63A,TMEM68,TME M87B,TNFAIP8L1,TNS4,TOMM40,TOMM70,TOR2A ,TPD52,TPD52L1,TRABD,TRAF4,TRIB2,TRIM2,TRI P11,TRMT61A,TRPC3,TSFM,TSG101,TSR1,TTC13 ,TTC27,TTC39C

Table 2D (cont'd)

		,TUBA1C,TUBB4B,TWF1,TXNDC9,TXNRD1,UAP1,UBA5,UBA6,UBQLN1,UBXN2A,UBXN2B,UCHL3,UCK2,UFL1,UNC13B,URB2,USO1,USP16,USP4,UTP14A,UTP14C,UTP18,UTP20,UXS1,VDAC2,VOPP1,VPS53,WAPL,WDR1,WDR36,WDR43,WDR75,WFS1,WIPI1,WWP2,XPNPEP1,XPOT,YIPF2,YIPF6,YKT6,YRDC,YWHAQ,ZBTB42,ZCCHC9,ZDHHC21,ZDHHC5,ZDHHC9,ZFP92,ZNF672,ZNF800

APPENDIX 3

SUPPLEMENTARY DATA FOR CHAPTER 3

Table 3A: RT-qPCR primer list

Gene	Species	Primer Sequences
CTGF	Human	(F)5'-CAG CAT GGA CGT TCG TCT G-3' (R)5'-AAC CAC GGT TTG GTC CTT GG-3'
CTGF	Mouse	F)5'-GGG CCT CTT CTG CGA TTT C-3' (R)5'-ATC CAG GCA AGT GCA TTG GTA-3'
RPL17	Human	(F)5'-ACG AAA AGC CAC GAA GTA TCT-3' (R)5'GAC CTT GTC TCC AGC CCC AT-3'
18S	Human	(F)5'-TGA TTA AGT CCC TGC CCT TTG T-3' (R)5'-TCA AGT TGC ACC GTC TTC TCA G-3'
18S	Mouse	(F)5'- GTA ACC CGT TGA ACC CCA TT-3' (R)5'CCA AAT CGG TAG TAG CG-3'
MiR-21	Human Mouse	UAGCUUAUCAGACUGAUGUUGA
U6	Human Mouse	GTGCTCGCTTCGGCAGCACATATACTAAAATT GGAACGATACAGAGAAGATTAGCATGGCCCC TGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTT
SMAD7	Human	(F)5'-TTC CTC CGC TGA AAC AGG G-3' (R)5'-CCT CCC AGT ATG CCA CCA C-3'
SMAD7	Mouse	(F)5'-GGC CGG ATC TCA GGC ATT C-3' (R)5'-TTG GGT ATC TGG AGT AAG GAG G-3'
36B4	Mouse	(F)5'- CAT CAC CAC GAA AAT CTC CA-3' (R)5'- TTG TCA AAC ACC TGC TGG AT-3'

APPENDIX 4

SUPPLEMENTARY DATA FOR CHAPTER 4

Table 4A: RT-qPCR primer list CUT&RUN

Gene	Species	Primer Sequence
pSTAT3	Human	(F) 5′-TGCCTCCCAAGTTTGCTAATGC-3′ (R) 3′-ACAATCTGTGCGTCATCCTTATCC-5′