1 Literature Review

1.1 Introduction

1.1.1 Introduction to endometriosis

Endometriosis [Gk, endon + metra, womb, osis, condition] is an oestrogen dependent condition with an estimated 10% prevalence in the general population (Olive and Schwartz 1993). It affects women of reproductive age, where endometrial glands and stroma from the eutopic endometrium are found in ectopic areas such as the ovaries, the posterior broad ligament, the anterior/posterior cul-de-sac, and the uterosacral ligaments (Zeitvogel et al. 2001). In some instances regions such as the lungs and kidneys may also be affected, although the incidence of these are rare (Rubin and Farber 1994). In all cases, the lesion sites are surrounded by increased vascularization and inflammation.

Postmenopausal women, who should have an absence of ovulation and retrograde menstruation, have also been diagnosed with endometriosis, but the incidence is very low with a range of approximately 2-5% (Punnonen et al. 1980; Ranney 1971). Postmenopausal diagnosis marks an aggressive form of the condition, with extremely high levels of aromatase cytochrome P450 expression (Bulun et al. 2000), an enzyme that encourages oestrogen synthesis and elevates breast cancer risk (Bertelsen et al. 2007). Other rare cases of endometriosis include women with tubal ligation and/or hysterectomy who should also theoretically be incapable of having retrograde menstruation. Because of these few cases, it has been proposed that other factors such as a genetic predisposition (Zondervan et al. 2007) and environmental toxins, may also be responsible for the condition (Kitawaki et al. 2002).

Endometriosis is usually referred to as a ‘disease,’ as it is usually seen in women with infertility that experience painful intercourse (dyspareunia), painful menstrual period (dysmenorrhoea), painful defecation during menses (dyschezia) and chronic fatigue (Varma et al. 2004). However, since endometriosis cannot be passed on from one person to another and is not contagious, it is more accurate to describe it as a condition, or more
accurately as an “epiphenomenon,” which is a by-product that arises from but does not influence another (Evers and Dunselman 1999).

In Australia, a 7% incidence rate of diagnosed endometriosis exists amongst 2400 individuals surveyed aged ≥ 18 years, with 12% of this occurring in women with known fertility problems (Clark 2006). An Australian cohort of twin pair families reveals a 2.34 risk ratio of developing endometriosis for siblings with first-degree affected relations (Treloar et al. 1999) whereby susceptibility genes in monozygotic twins are concordant for endometriosis (Hadfield et al. 1997).

In reality the exact incidence cannot be known, for endometriosis has a benign aetiology, usually requiring an invasive laparoscopic procedure to accurately diagnose a patient. Laparoscopy utilises a thin telescope that directly visualize lesions, removes them by surgery and confirms by biopsy the presence of glands and stroma. Histological examination provides a complementary technique to confirm the presence of endometriosis and exclude more life threatening pathologies such as malignancy. In fact, a huge lag time can exist between first symptom and successful diagnosis, with estimates of 7.96-11.73 years before an accurate and definitive diagnosis (Hadfield et al. 1996). In Australia, the delay in diagnosis is approximately 8 years (Treloar et al. 2002), where some women with advanced endometriosis can remain asymptomatic, causing many to remain undiagnosed, whereby the only indication of endometriosis is the cyclicity of its symptoms. The asymptomatic nature in these women contributes to the difficulty in formulating a suitable and effective ‘one-size fits all,’ treatment regimen, as well as the creation of guidelines for a ‘full-proof,’ diagnosis. Unfortunately, by the time most women are diagnosed, they have usually suffered many years of pain and discomfort and have taken various medications to treat other conditions with very similar symptomology.

It is advisable that a thorough history and examination be conducted, with a particular emphasis on establishing the chronicity of pelvic pain symptoms, to ensure that delays in
diagnosis and treatment are prevented. Early diagnosis and treatment may prevent more debilitating outcomes in the future, such as infertility and anatomical abnormalities (Dovey and Sanfilippo 2010).

According to the American Society of Reproductive Medicine (A.S.R.M 1997), endometriosis has varying severity that can be divided into two main forms: the mild (Stage I and II) and the severe form (Stage III and IV). Refer to Appendix 1.2 for the A.S.R.M revised classification of endometriosis for a matrix to determine the severity of disease. Depending on the stage of the disease, a number of treatments can be given varying from powerful oral contraceptives to surgical ablation. However, these measures do not provide long lasting solutions, as endometriosis can reappear even after many years of being symptom free, supporting the idea the condition is present in situ even after treatment measures are taken.

Severe forms of endometriosis that cause chronic pain are seen in rectovaginal nodules due to the close apposition of lesions to nerves (Anaf et al. 2000), leading to a decreased quality of life where repeated and often unsuccessful treatments can cause lesion recurrence and unwelcoming symptoms. These symptoms range from emotional stress and increased masculinity associated with the use of testosterone derivatives. Moreover, the associated pain and discomfort are exacerbated by peritoneal inflammation, deep infiltration, tissue damage, adhesion, fibrosis and the accumulation of menstrual blood that prohibits surrounding tissue movement.

1.2 The normal endometrium

1.2.1 The female uterus

The female uterus is made up of three layers:

- Endometrium
- Myometrium
- Serosa
The endometrium makes up the inner layer of the uterus and is the region that will be examined most extensively in this project, as it is the site where endometrial cells thought to cause endometriosis originate. The endometrium is divided into two zones, the basalis and functionalis. The functionalis layer is sloughed off during menstruation, and the basalis, which remains intact during this period, aids in the regeneration of the functionalis. The functionalis is the region that responds to fluctuating oestrogen and progesterone levels and progressively becomes thicker during the proliferative and secretory phase of the menstrual cycle, in preparation for a possible embryo implantation.

The myometrium is the thickest layer of the uterus and makes up its intermediate layer. It consists of smooth muscle tissues that contract during the shedding of the endometrial lining at menstruation, and also during childbirth.

The serosa makes up the final outermost layer of the uterus that is continuous with connective tissue structures such as ligaments, and provides mechanical support to the uterus.

1.2.2 Endometrial changes during the menstrual cycle

The endometrium of women with normal ovulatory cycles, continually responds to changes in steroid hormones and they influence its extensive growth and remodelling potential. The female menstrual cycle can be divided into three phases: the menstrual, proliferative and secretory phase.

On days 1-5, the menstrual phase begins, as embryo implantation has not occurred. The levels of oestrogen and progesterone drop dramatically, creating an inflammatory, apoptotic environment, where extracellular matrix (ECM) proteins are broken down and menstruation is commenced by several genes including plasminogen activator, urokinase (PLAU) and tumour necrosis factor-alpha induced protein 2 (TNFAIP2) (Gaide Chevronnay et al. 2009). Consequently, the functional layer degenerates and detaches from
the uterine wall due to proteins such as MMP’s, IL-8 and MCP-1 that regulates ischaemia, and is shed out through the vagina. Menses is characterized by endometrial shedding due to proteolysis and ischaemia (Molina 2010). Proteolysis of glandular and stromal cells involves enzymes that first collect in lysosomes post-ovulation, and are released after lysosomal membrane breakdown as a response to decreasing oestrogen and progesterone (Molina 2010).

The second part of menses involves ischaemia, whereby blood vessel vasoconstriction within the endometrium leads to capillary vessel rupture and consequently menstrual bleeding (Molina 2010). Steps involved in the ischaemic process include matrix degradation by leukocytes that secrete MMP’s, adding to the IL-8 and MCP-1 produced prior to menses (Arici et al., 1995). Furthermore, increased levels of prostaglandin F2α during the late secretory phase activate lysosomal secretion of acid hydrolases, enhancing myometrial contractions and endometrial cell expulsion (Molina 2010).

ECM renewing proteins such as collagen type I alpha 1(COL1A1) and tissue inhibitor of metalloproteinase 1 (TIMP1) present within the menstrual effluent, may allow endometrial fragments to survive at ectopic sites (Gaide Chevronnay et al. 2009), in addition to angiogenic proteins such as the monocyte chemotactic peptide-1 (MCP-1) (Arici et al. 1995) and interleukin-8 (IL-8) (Kyama et al. 2006).

During days 6-13, the proliferative phase occurs. During this time, the follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels from the anterior pituitary gland are increasing, in preparation for the LH surge during ovulation (Figure 1.1). In addition, elevating oestrogen levels promotes the LH surge via positive feedback, activating granulosa cells to release cytokines (i.e. Tumour necrosis alpha, TNF-α), progesterone and prostaglandins (Wang et al. 1992). These secreted proteins also initiate the release of matrix metalloproteinases (MMPs) and their associated TIMPs from granulosa and theca cells, creating the balancing act that mimics an inflammatory response and ECM
remodelling (Curry and Osteen 2003). Infiltration by leukocytes, as well as changes to the blood vessel architecture and adhesion molecules are also seen (Rossi et al. 2005).

During the proliferative phase, progesterone and prostaglandin aid in ovulation, and along with the LH surge, begin the luteinization process, where new blood vessels are created with the help of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Kobayashi et al. 2001).

In this phase, the released FSH stimulates the growth of follicular cells within the ovary. As a follicle develops, there is a steady rise in oestrogen that leads to an increase in endometrial cell proliferation and thickness. Progenitor cells located in endometrial cells aid in endometrial regeneration as stem cells found within the basalis provides the proliferative capacity for the functionalis to rebuild its structure (Gargett and Masuda 2010). A genetic profiling study shows that a number of genes involved in cell cycle regulation are expressed during this period, taking part in endometrial replication, proliferation and remodelling (Kuokkanen et al. 2010). Remodeling and increased vascularity stimulates epithelial and stromal cells via various growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor-alpha (TGFα) (Chan et al. 2004). The endometrium becomes thicker, reaching about 2-3 mm in thickness at the end of the phase (Figure 1.2). The glands within the endometrium are relatively straight during this time and are aligned with simple columnar epithelial cells, as well as a thick, compact connective tissue stroma. The nuclei within the glands begin to crowd together as increased cellular and mitotic nuclear activity begins within the glandular and stromal cells, leading to a thickening of the endometrium. At this time, no glycogen and mucus secretions can be found within the lumen. In a standard 28-day cycle, LH surge occurs at day 14 with ovulation 36-42 hours later (Figure 1.1).

Within days 15-28, the secretory phase commences and the endometrium undergoes a dramatic change in appearance that cause glands to become more tortuous and dilated, and
the lumen to become larger and filled with glycogen rich secretions, in preparation for a possible embryo implantation. The stroma becomes oedematous, cells more rounded and irregularly arranged the endometrial lining also reach its maximum thickness. At post-ovulation, the endometrium undergoes cellular differentiation, inhibits further mitotic activity and DNA synthesis, as a response to rising progesterone. The endometrium also further inhibits oestrogen action by down-regulating their receptors within the epithelium and by activating its metabolising enzyme 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) (Cheng et al. 2007). These changes create the ‘window of implantation,’ which is an optimal environment for a potential embryo, whereby progesterone causes stromal cell decidualization and the differential expression of 238 genes that includes growth differentiation factor 15 (GDF15) and the angiogenin, ribonuclease, RNAse A family, 5 (ANG) (Diaz-Gimeno et al. 2011). Moreover, MMPs are decreased during the secretory phase due to a progestogenic effect (Osteen et al. 1994) and later rises at the end of the phase due to falling steroids (Salamonsen et al. 1997). It is thought that the increase in MMP/TIMP ratio at the end of the secretory phase may enhance the invasive potential of endometrial cells to develop at ectopic sites (Szamatowicz et al. 2002).

There are many other factors involved in mediating endometrial cell growth and development ranging from growth factors, cytokines, cell cycle mediators and immune regulators. Examples include the epidermal growth factor (EGF) and its receptors that respond to changes in oestrogen levels (Huet-Hudson et al. 1990) and insulin-like growth factors (IGFs) which are produced by uterine tissues (Giudice et al. 1993) that are important in their development (Liu et al. 1993). Furthermore, cyclin D1 is involved in cell proliferation (Karin 2006) and transforming growth factor beta (TGF-β) contains an immunosuppressive function for any increase in CD4+ levels (Ouellette et al. 1997).
Figure 1.1: The human menstrual cycle. (A) Changes in pituitary gland hormones; (B) oocyte development with changes in hormone levels; (C) changes in ovarian hormone levels and (D) changes in endometrial cell thickness.

(Gilbert). “Developmental Biology Online: Hormones and Mammalian Egg Maturation.”
Figure 1.2: A schematic view of the endometrial changes in the baboon (Papio anubis) and women during the menstrual cycle derived from (Gilbert). “Developmental Biology Online: Hormones and Mammalian Egg Maturation;” Kraemer et al. 1977 and Christensen et al. 1995)
1.3 Human endometriosis

1.3.1 Definition of endometriosis

During the normal human menstrual cycle, endometrial cells respond cyclically to circulating hormones such as progesterone and oestrogen, where they regenerate in preparation for embryonic implantation, and degenerate as menstrual effluent at the end of the menstrual cycle. Endometriotic cells similarly respond in a cyclic manner to progesterone and oestrogen, however, as they are found at ectopic locations, they cannot be shed as menstrual effluent through the vagina, and thus progressively increase in size, leading to local inflammation and haemorrhage.

As briefly mentioned, endometriosis is a painful condition for which there is currently no effective cure. For instance, surgical ablation of lesion areas does not prevent recurrence in the future, and hormonal therapy has unsatisfactory side effects such as increased masculinization, although is effective in suppressing further endometrial cell growth.

Since 1996, three heterogeneous clinical categories have been created to define the different types of endometriosis (Donnez et al. 1996).

1) Peritoneal endometriosis
2) Ovarian endometriosis (also known as ‘chocolate cyst,’ due to the dark coloured blood from invading endometrial tissues in the ovaries)
3) Rectovaginal endometriosis (also known as ‘rectovaginal adenomyosis’ or ‘rectovaginal adenomyoma.’)

As with other enigmatic, multifactorial conditions such as heart disease and diabetes, the aetiology of endometriosis is unknown. However, its origin has been categorized into distinct theories:
1.3.2 The Origin of Endometriosis

1. Embryonic cell metaplasia theory: First described by William Wood Russell in 1899, reveals how the müllerian tract gives rise to the female reproductive tract during embryonic development (as revised by (Longo 1979)). It is thought that these inactive embryonic cells are activated during puberty as a response to increasing oestrogen levels that encourage their growth.

2. Retrograde menstruation theory: First proposed by Sampson as the movement of viable endometrial cells to ectopic locations (Sampson 1921), where the majority of women experience retrograde menstruation but not all develop endometriosis. This theory suggests that a small number of women have compromised immune systems that are unable to effectively clear implanted lesions at ectopic sites. Several theories exists as to how these lesions are formed in the first place, ranging from a prolonged uninterrupted regular menstruation (Moen 1987), to toxins (Rier et al. 1993), to the more recent role of hereditary factors (Treloar et al. 2002).

The validity of Sampson’s theory is questionable, as there are some mechanisms that simply do not fit, some of which are listed below:

- Most reproductive age women have a degree of menstrual reflux but yet only some develop endometriosis (Wheeler 1989). Moreover, the location of endometriotic lesions have a reproducible pattern of distribution and do not seem to be more widespread in older women (Punnonen et al. 1980; Ranney 1971), who should theoretically have had more endometrial cell reflux over the course of their reproductive life, with regular menstruation.

- Only 717 out of 53,000 probes on a whole human deoxyribonucleic acid (DNA) microarray chip were changed two-fold in the ectopic than the eutopic endometrium.
This questions Sampson theory that endometriotic cells result from auto transplanted eutopic endometrial tissues (Eyster et al. 2007).

- Endometriosis located at distant locations such as the lungs and skin cannot be adequately explained by Sampson’s theory. These lesions may be attributed to lymphatic transport (Ueki 1991).

- And finally, surgical removal of endometriotic lesions does not prevent adhesion recurrence and does not provide a complete cure (Busacca et al. 2006).

1.3.3 The cause of endometriosis

Some theories of origin described above also take part in its cause. Below is a list of current opinions as to the cause of endometriosis.

1) Deregulation of candidate endometriosis genes such as an excess oestrogen production caused by genes involved in the metabolism of oestradiol (E₂), where cyp19 encoding P450 aromatase is found in ectopic lesions (Noble et al. 1996). The activation of the wingless int (Wnt) (Hou et al. 2004) and rat sarcoma (Jabbour et al. 2005) genes in the eutopic endometrium of women with endometriosis that activate oncogenic pathways, oestrogen and prostaglandin formation respectively, amongst others. The down-regulation of the integrin transcript that would prevent embryo attachment to its surrounding endometrial tissues, may lead to infertility in women with endometriosis (Kao et al. 2003). The 100-fold increase in kallikrein that may allow ECM proteolysis as well as angiogenesis and a dysregulation of B61, an ephrin family member that is known to have angiogenic and migratory capabilities (Kao et al. 2003).

2) Retrograde menstruation as described previously, can be attributed to hereditary factors (Treloar et al. 1999), environmental toxins such 2,3,7,8-tetracholrodibeno-
p-dioxin (TCDD or dioxin) (Rier et al. 1993) or a compromised immune system that cannot readily dispose and prevent ectopic cell implantation.

3) Coelomic metaplasia: Endometriotic tissues originate from stem cells that can be activated by menstruation, toxins and/or immune related factors.

4) Hereditary factors: Familial correlation reveals that mothers affected with endometriosis are likely to have daughters with the condition and female siblings of those with endometriosis are at a greater risk of developing ectopic lesions compared with the control population (Treloar et al. 1999). A susceptibility locus on 10q26 in sib-pairs from 1176 families (Treloar et al. 2005) and polymorphisms involving VEGF (Bhanoori et al. 2005) amongst others, reveal that hereditary mechanisms are likely to play a role in the prevalence of endometriosis.

5) Transplantation to distant sites such as the lungs and skin may be due to lymphatic transport (Ueki 1991), where the most common location of extra pelvic lesion is seen in the abdominal wall (Ideyi et al. 2003). Recent theories stipulate that since endometrial cells have inherent progenitor behaviour, akin to stem cells, it is possible that they support endometrial cell regeneration and survival at ectopic locations (Schwab et al. 2008).

6) Transplantation after incisional scars: Some patients are susceptible to ectopic lesion development after a caesarean procedure or episiotomy (Gunes et al. 2005); hysterectomy (Kill et al. 2011); laparoscopy (Kumakiri et al. 2010) or appendectomy (Akbulut et al. 2010).

7) Environmental factors: A range of environmental influences such as hormones contained in food products due to the environmental pollutant dioxin, an organochloride compound found in pesticides (Clark et al. 1992). In the case of
dioxin, it is thought that TCDD disrupts progesterone’s inhibition of the MMP system, allowing ECM degradation (Bruner et al. 1997) whilst deregulating progesterone’s suppressive effect (Igarashi et al. 2005). However, conflicting results reveals no strong evidence for dioxin mediated endometriosis, as seen by a 31/100 score under strict epidemiological criteria that looks at nine different categories ranging from strength, biological gradient to analogy, with a maximum score being 100 (Guo et al. 2009). In addition, iron has a potential oxidative, aggravating role as a number of endometriosis related genes coincide with iron regulated ones such as those involving the cell cycle, growth factors, hormones, adhesion molecules and detoxification (Kobayashi et al. 2009).

8) Excess oestrogen production that occurs in ectopic lesions (Bulun et al. 2000) and oestrogen in birth control medications can exacerbate and encourage further lesion growth, where oestrogen can build up in fat and liver cells and over time is unable to be eliminated from the body. This is the main reason why combined oral contraceptive (COC) pills containing synthetic oestrogen and progestin compounds are used to manage endometriosis (Meresman et al. 2002). In addition, when endometriotic cells are exposed to hormones such as prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), that is up-regulated in endometriosis (De Leon et al. 1988) it promotes the binding of steroidogenic factor-1 (SF-1) to the promoter of genes of aromatase 450, leading to increased oestrogen synthesis (Zeitoun et al. 1999). Further activation of PGE\textsubscript{2} via cyclooxygenase-2 (COX-2) and VEGF action that are also seen in endometriotic tissues, may lead to a subsequent increase in oestrogen production (Tamura et al. 2002).

9) Progesterone resistance that leads to a dysregulated induction of the oestrogen metabolising enzyme HSD17B2, can elevate oestrogen levels in endometriotic tissues, (Zeitoun et al. 1998) as the enzyme is unable to convert oestrogen to its less active estrone form (Cheng et al. 2007). This mechanism may be enhanced by
preferential hypermethylation that renders PR-B inactive (Wu et al. 2006) where ectopic lesions have decreased progesterone responsive genes such as insulin like growth factor binding protein 1 (IGFBP1) (Lee et al. 2009) and homeobox protein A10 (HOXA10), that affect endometrial cell differentiation (Kim et al. 2007) as seen in the baboon model. Furthermore, the combination of a low ERα and ERβ ratio and its subsequent PR inhibition in endometriotic stromal cell cultures, may explain progesterone resistance in women who are PR-B deficient (Xue et al. 2007).

10) Epigenetic phenomena that cause impaired methylation (Wu et al. 2007), histone acetylation (Sun et al. 2003) and microRNAs (miRNA) expression (Ohlsson Teague et al. 2009) leading to chromatin remodelling, deregulated aromatase control and infertility. Down-regulation of various miRNA can lead to an inflammatory and neoangiogenic environment (Chakrabarty et al. 2007) and promotion of endometrial cell proliferation (Ohlsson Teague et al. 2009).

11) Enhanced neovascularization processes can be seen in and around implanted tissues as confirmed by microscopic studies (Nisolle et al. 1993), where VEGF was found particularly within epithelial cells (Shifren et al. 1996). Stromal cells can also exhibit VEGF (Shifren et al. 1996) and along with neutrophils and macrophages (Mueller et al. 2000a) all contribute to endometriotic angiogenesis.

Also, oestrogen response element was discovered -1525 bp upstream of VEGF mRNA (Mueller et al. 2000b) that allows the steroid to enhance the angiogenic activity of VEGF in an in vitro model (Shifren et al. 1996).
1.3.4 Diagnosing endometriosis

Due to the rising health care costs and risks associated with repeated surgery, as well as the deleterious effect on a woman’s ovarian reserve, the development of better predictive methods for recurrent endometriosis is warranted (Candiani et al. 2005).

Women with endometriosis have a benign pathology that cannot readily be diagnosed, with non-specific symptoms ranging from painful cramps and general malaise that can be mistaken for a number of different conditions. The only accurate method of diagnosis is by using laparoscopy for small lesions, as well as imaging techniques such as magnetic resonance imaging (MRI) and ultrasound for larger endometriotic lesions such as nodules and cysts (Guerriero et al. 1995). This is especially pertinent in rare umbilical endometriosis lesions, whereby transvaginal and abdominal ultrasound, with magnetic resonance imaging is needed to show the extent of ectopic cell infiltration and to exclude pelvic endometriosis (Mechsner et al. 2009).

Endometriosis can only be definitively diagnosed after lesion biopsies and microscopic investigation that can confirm the laparoscopy and/or imaging techniques. Biopsies are extremely important, especially in cases such as ureteric endometriosis (Berlanda et al. 2009) as it is often misdiagnosed, due to its non-specific presentation that may also be attributed to other differential diagnoses such as granulomas or abscesses (Bektas et al. 2010).

Genomic microarray technologies used to ascertain and isolate susceptibility genes that may one day prove to be effective markers have been used (Matsuzaki et al. 2004). However, no one absolute marker is available, except for the highly documented cancer antigen-125 (CA-125). But this marker is neither specific as it is also seen in ovarian cancer (Kim et al. 2008) nor indicative of the varying forms of endometriosis, which can range in severity, lesion type and level of inflammatory and invasive capacity i.e. inactive or active (A.S.R.M 1997; Kennedy et al. 2005).
Macroscopically, endometrial lesions can have a 'red or bluish' pigmentation, fibrous adhesions, and a 'powder burn' appearance due to the deposition of hemosiderin-laden macrophages (Rubin and Farber 1994), which is a response to recurrent bleeding induced by changing hormone levels. ‘Subtle’ lesions include red implants and serous or clear vesicles, whereas white plaques and yellow-brown peritoneal discoloration signifies other typical endometriosis. These ectopic implants respond to the same progesterone and oestrogen levels that act on the uterine endometrium during the menstrual cycle. However, unlike the endometrial cells from the eutopic endometrium that can pass out through the vagina as menstrual flow, endometriotic implants remain at their ectopic locations causing internal bleeding, inflammation and the breakdown of blood and tissues. These lesions swell in size prior to, or during menstruation, causing a wide spectrum of symptoms ranging from debilitating pain, menorrhagia, and infertility (Zondervan et al. 2002) and the formation of scar tissues and adhesions (Daniel and Wilson 2008).

Endometriosis related infertility has multiple causes that include the TNF-\(\alpha\) rich peritoneal fluid inhibition of sperm-zona pellucida interaction (Faber et al. 2001), reduced ovarian function (Barri et al. 2010) and raised immune cell numbers within the endometrium (Schulke et al. 2009) amongst others. The aforementioned markers can be used to establish fertility status in women with endometriosis, by using techniques such as proteomics (Siristatidis 2009).

Evidence also suggests that endometriosis may play a role in initiating ovarian cancer because of its comparable invasive and migratory activity to carcinoma cells (Sotnikova et al. 2002; Zeitvogel et al. 2001). This theory is substantiated by an odds ratio of 1.73, (95% confidence interval 1.2-2.4) as an increased susceptibility for women with endometriosis to develop ovarian cancer (Ness 2003). Additionally, a large retrospective study involving 6,398 women in Japan indicates that ovarian cancer is more likely to develop in those with concurrent ovarian endometriomas (Kobayashi et al. 2007) leading to endometriosis-associated ovarian cancer, (EAOC). The Endometriosis Association also states that women
with endometriosis have a 2.5 times higher susceptibility for non-Hodgkin’s lymphoma and a 6 times risk for breast cancer compared with the general population (Duczmann and Ballweg 1999). Moreover, women diagnosed at approximately ≥ 40 years of age have an even greater risk for breast cancer, compared to those diagnosed at a younger age (Bertelsen et al. 2007).

1.3.5 Current therapies
As approximately 40-45% of patients have recurrent endometriosis, 5 years after their initial surgery, more efficacious treatments are required to prevent relapse (Garry et al. 2000). The first treatment of choice for endometriosis, especially in symptomatic deeply infiltrating types, is resection by surgical ablation or excision. However, some lesions are located in highly delicate areas that are difficult to access such as the rectosigmoid area (Duepree et al. 2002), and/or are very small in size and remain undetected by endoscopic investigations. Thus the combination of physical resection, as well as hormonal treatments, can provide a more comprehensive management for the patient. This is seen in cases of abdominal wall endometrioma, where surgery with wide excisional margins is the recommended intervention with concurrent hormonal therapy (Wang et al. 2003). This is especially important for endometriosis that is chronic in nature, with a high recurrence rate, where repeated, invasive therapies can unnecessarily inconvenience patients, causing them stress and anxiety.

Current drug treatments change the hormonal balance of the menstrual cycle by producing a chronic anovulation, a pseudopregnancy by endometrial decidualization, or a pseudo-menopause by endometrial atrophy as seen in amenorrhea (Cahill 2002). Drug treatments can take the form of combination hormone therapy, as seen in oral contraceptive pills or non-steroidal anti-inflammatory drugs (NSAID).

NSAIDs are an appropriate first line treatment for women with endometriosis, wishing to conceive, as they do not have a contraceptive action. However, a Cochrane Review
discovered that no conclusive evidence exists that the anti-inflammatory action of NSAIDs can provide effective pain management, in women with chronic endometriosis (Allen et al. 2009).

Other treatments such as PR antagonists (Kobayashi et al. 2010) and selective progesterone receptor modulators (SPRMs) (Chwalisz et al. 2005) are currently being clinically tested for endometriosis and cancer research, for their potential control of inflammatory and pain mechanisms.

The aforementioned drugs aim to reduce the size, the growth rate and the pain associated with endometrial implants, but cannot eliminate the disease or improve fertility rates of women with endometriosis. This is shown by when a placebo or an active drug is given to affected patients and withdrawn after treatment, the same rate of pregnancy was achieved (Cahill 2002).

The traditional therapies available to patients with endometriosis are listed in Appendix 1.1. A combination of these treatments may be necessary for the management of the condition, especially for women with the severe form of endometriosis. However, caution must be taken as progestin, danazol and gonadotrophin agonists may benefit only women with Stage I or II endometriosis (A.S.R.M 1997), with about 80-90% of women gaining pain relief (Cahill 2002). Although these drugs are effective in relieving pain, they give unwanted androgenic and menopausal side-effects that can potentially reduce compliance (Bergqvist et al. 1998).

Other emerging treatments for inflammatory like conditions and cancer involve the ubiquitin-proteasome system which utilize the proteasome inhibitor bortezomib for multiple myeloma (Tansey 2004). Since endometriosis has inflammatory and metastatic characteristics, where inflammation may even mediate ovarian cancer risk (Ness et al.}
manipulating the ubiquitin-proteasome system may prevent the formation of ectopic endometrial lesions.

Recently, a randomised placebo-controlled trial that examined the effect of Infliximab, a TNF-α antagonist on women with rectovaginal and advanced stage endometriosis revealed no conclusive evidence that the drug was superior to placebo (Koninckx et al. 2008). In this study, no improvement in dysmenorrhoea and dyspareunia was seen with Infliximab, however the visual analogue pain score (Putnam et al. 2007) decreased in the placebo group (Koninckx et al. 2008).

Additionally, a comprehensive systematic study from the Cochrane Review recently examined the efficacy of chinese herbal medicine (CHM) in endometriosis (Flower et al. 2009). The report concluded that CHM offered similar symptomatic relief compared to the anti-progestin gestrinone post-surgery (95.65% vs. 93.87%; RR 1.02, 95% CI 0.93 to 1.12), similar to the study involving oral CHM and danazol (56.3% vs. 11.1%; RR 5.06, 95% CI 1.28 to 20.05) (Flower et al. 2009). In addition, a combination of oral CHM and enema versus danazol revealed a greater improvement in dysmenorrhea pain score (MD -2.90, 95% CI -4.55 to -1.25; P < 0.01) and lesion regression (RR 1.70, 95% CI 1.04 to 2.78) but no improvement in vaginal, rectal or lumbosacral related pain (Flower et al. 2009). The review recommends that more randomised clinical trials need to be conducted to compare various types of CHM/techniques with proven treatment modalities to determine its efficacy.

Interest on inhibitors of aromatase (Verma and Konje 2009) and COX-2 (Olivares et al. 2008), as well as progesterone (Shimizu et al. 2009) is an active area of research, as these modulators provide symptom relief. In the future, postmenopausal (Takayama et al. 1998) and premenopausal women (Shippen and West 2004) with persistent pain symptoms despite surgical and medical treatment of endometriosis, may be given aromatase inhibitors that prevent the conversion of androstenedione and testosterone to estrone and oestradiol.
Other future treatments may include anti-angiogenic and anti-vascular agents that may prevent lesion establishment and development (Ricci et al. 2011), as well as sophisticated gene therapy techniques that introduce promoters specifically targeting lesions (Dabrosin et al. 2002). Furthermore, blocking sex-steroid production due to the discovery of new LH-RH antagonists may also be possible (Chen et al. 2008a). However, further randomised clinical trials are needed to establish the efficacy of these patient tailored approaches, as current knowledge is still derived from experimental models.

Finally, as women with endometriosis can suffer from infertility, a recent review recommends that those with severe cases undergo IVF treatment in addition to surgery, to maximise the number of mature oocytes and lessen their discomfort (Dechaud et al. 2009).

1.3.6 Surgical treatment of endometriosis

A range of surgical treatments exists depending on the severity of ectopic lesion areas.

1) Laparoscopy: Usually the first surgical treatment of choice, that utilizes the ablation of lesion areas.

2) Laparotomy: Often used if extensive surgery is required to ensure that a woman’s reproductive potential is at the very least, not further compromised, or at best preserved.

3) Hysterectomy: The complete surgical removal of the uterus and surrounding tissue which may/or may not include the fallopian tubes and ovaries (salpingo-oophorectomy).

4) Bowel resection: The surgical removal of lesion areas within the bowel.

5) Neurectomy: The surgical removal of nerves to the uterus located near the sacral region. Women with chronic pain associated with endometriosis have abnormal nerve structures within their pelvis (Tokushige et al. 2007), as the nerves within the
uterovaginal plexus can be injured after excessive straining with defecation and a
difficult pregnancy (Quinn 2005). The irregular pattern that occurs with nerve
reinnervation impinges on the blood vessels of various pelvic organs that may lead
to dysmenorrhoea and chronic pain (Anaf et al. 2000), that is exacerbated by an
impaired uterine peristalsis.

6) Embolization: Purposeful blood vessel occlusion to prevent blood flow to painful
lesioned areas are currently under investigation (Kang et al. 2009).

1.4 The baboon animal model (Papio anubis)

1.4.1 Endometriosis and the baboon

There is currently no perfect cell culture model that mimics the extensive cross talk
between different cells of the endometrium. The most commonly used animal to
understand human uterine biology is the mouse model, particularly due to the existence of
knockout mice that allows the examination of gene deficiencies in endometriosis related
research (Tranguch et al. 2007). However, using non-human primates in endometriosis
research is a better way of establishing the cause and effect of endometrial cell
implantation and endometriosis development that other animal models such as rodents
can’t provide, due to factors such as a lack of menstruation and wide phylogenetic variation
(Zondervan et al. 2004).

The baboon, like humans, is a continuous breeder that has menstrual cycles throughout the
year, which continue in captivity. It is used as an ideal non-human model for reproductive
based studies, for it shares many similarities with humans in terms of the cyclic changes
that occur within the endometrium during the menstrual cycle, and the types of proteins
expressed. Fazleabas and colleagues thoroughly describe the many advantages to using the
baboon model (Fazleabas et al. 2002), with the greater expense involved as opposed to
other species being the only major disadvantage.
Baboons are a proven animal model for other areas of medical research such as cardiovascular research (Lovering et al. 2007; Shi et al. 2007), endoscopic surgery (Martinez-Serna et al. 2000; Oberg et al. 1999) endocrinology (Beehner et al. 2006; Green et al. 2000), teratology (Moore et al. 2007; Tarara 1984) and toxicology (Schlabritz-Loutsevitch et al. 2004). The similarities between baboons and humans include menstrual characteristics, embryo implantation and foetal development, which make the non-human model a valuable resource. Baboons and humans are also phylogenetically close (Fazleabas et al. 2002), which means any genetic influence relevant to endometrial cell development would likely mimic or accurately resemble each other.

Below is a description by Fazleabas and colleagues that thoroughly describes the many advantages in using the baboon animal model.

As thoroughly described by Fazleabas and colleagues (Fazleabas et al. 2002), the many advantages in using the baboon animal model include its phylogenetic closeness to humans, including its similar reproductive anatomy and physiology; its proven role in other branches of medical research such as cardiovascular and endoscopic surgery; its continuous ability to breed, even when in captivity; its stronger constitution to repeated sampling compared to smaller primates, as well as the presence of spontaneous endometriosis at varying stages.

1.4.2 Baboon histology

Much like women of reproductive age, the baboon menstrual cycle can also be divided into three phases: the proliferative, secretory and the menstrual phase. For the purpose of this study, the three stages will be referred to as the follicular, luteal and menses phases respectively, as these are the conventional nomenclature for baboon endometrial related studies (Figure 1.3). The days and histological changes referred to in this review, is a combination/modification of the study by Kraemer and colleagues, which begin on day -14 menses and ends on day +18, late secretory phase (Kraemer et al. 1977), and Christensen
and colleagues, with day 0 as the start of menses and day +30 as the end of luteal phase (Christensen et al. 1995).

![Diagram illustrating treatment groups and sampling time points for the baboon. The days indicated below the lines are the times at which tissue was obtained. M = menses; EF = early follicular; LF = late follicular; ML = mid-luteal; LL = late luteal (Christensen et al., 1995).](image)

**Figure 1.3.** Diagram illustrating treatment groups and sampling time points for the baboon. The days indicated below the lines are the times at which tissue was obtained. M = menses; EF = early follicular; LF = late follicular; ML = mid-luteal; LL = late luteal (Christensen et al., 1995).

On days 0-5 menses begins, for embryo implantation has not occurred (Figure 1.2). The surface epithelial layer detaches from the uterine wall and is discharged through the vagina. Remaining glandular cells reveal a degree of degeneration as well as tortuosity, where an absence of secretory material is seen in columnar epithelial cells. The presence of erythrocytes and leukocytes is evident along the sloughed off surface.

During days 6-14, the follicular phase begins (Figure 1.2). Glandular epithelial cells change from being cuboidal and pseudostratified in appearance with a very limited, almost non-existent degree of mitosis, increasing in cell size and shape into a mixture of low to tall columnar epithelium. Stromal cells also undergo some changes during the follicular phase, first possessing a moderately dense architecture with infrequent mitoses and gradually increasing in their degree of cell division and density. Day 15 marks the window of ovulation.
At days 16-30 the luteal phase commences. Glands appear relatively coiled containing basal and mid-cytoplasmic vacuoles of secretion, becoming progressively tortuous in appearance with their characteristic sinuous path of tall columnar and monostratified epithelium. Stromal cells initially appear dense with few mitoses and spiral arteries, gradually becoming loose in structure, increasing in oedematous secretion and predecidual transformation. The endothelium of spiral arteries also becomes more prominent and a number of leukocytes appear between the stromal cells.

1.4.3 Inducing endometriosis in the baboon

Endometriosis can be induced in the baboon by physically implanting viable endometrial cells by intraperitoneal inoculation. A detailed description of this procedure is described by Fazleabas and colleagues (Fazleabas et al., 2002) whereby a Unimar pipelle (Cooper Surgical, Shelton, Connecticut, USA) is used to isolate baboon endometrial cells at late follicular (Tabibzadeh et al. 1995), mid luteal (ML) and menstrual phases. ML occurs between days 9 and 11 post-ovulation, representing the interval of window of uterine receptivity in this primate (Hastings and Fazleabas 2006). On the day of laparotomy, a baboon’s menstrual cycle phase is determined by measuring their oestradiol and progesterone serum levels, observing cycle history as well as their level of arousal through sex skin changes, marking periods of greater reproductive potential. Based on this information, LF, ML or menstrual phase endometrial cells are then placed into the peritoneum.

1.5 Ubiquitin and the proteasome

1.5.1 Ubiquitin structure and function

The ubiquitin-proteasome pathway is a highly conserved proteolytic mechanism within yeast to mammals, responsible for degrading short, regulatory proteins involved in signal transduction (Bebington et al. 2001) within the cytoplasm and the nucleus (Krappmann and Scheidereit 2005; Lindsten and Dantuma 2003). The process is adenosine triphosphate (ATP)-dependent, involving various types of ubiquitin that are used to recognise and mark
proteins for degradation within the proteasome (Davy et al. 2001). A loss in regulatory function, can lead to aberrant accumulation of ubiquitinated proteins (Goldberg 2005).

Ubiquitin is a 76 amino acid highly conserved protein found in eukaryotic organisms. The ubiquitin gene is translated into fusion proteins that can be one of two forms: a ubiquitin and ribosomal protein form or a linear ubiquitin repeat. Once these proteins are translated, the ubiquitin C-terminal hydrolase can cleave the C-terminal ends of ubiquitin within the fusion proteins, allowing the release of individual ribosomal and ubiquitin subunits.

Ubiquitin contains many different isoforms, where a polyclonal antibody for example can detect either an enzyme-1 (E1), an ubiquitin-activating enzyme (Schwartz and Ciechanover 1999), the ubiquitin protein conjugates or free ubiquitin monomers. The ubiquitin antibody can also detect homologous proteins, such as the interferon stimulated gene 15 (ISG15), also known as the ubiquitin cross reactive protein (UCRP).

Ubiquitin's main function is to regulate the turnover of specific proteins such as those that are misfolded, mutated or short regulatory proteins. Once proteins are no longer required by the body (e.g: cyclins) or are detrimental to the body (e.g: viruses), they are tagged by ubiquitin and sent to the proteasome for degradation. This is an important mechanism, for it ensures that harmful or unnecessary proteins are eliminated from the body to maintain homeostasis.

The ubiquitin system has several ways of regulating protein turnover by recognising the degradation sequences on the target protein. Further research is being conducted in discovering other recognition sequences (Glickman and Ciechanover 2002). The following list is by no means exhaustive for it merely illustrates the possibilities of how proteins may be targeted.
1) N-degron sequence.
2) PEST sequence. (P = proline; E = glutamic acid; S = serine and T = threonine).
3) Amino acid residues outside the hydrophobic core.

The N-degron rule, first discovered by Varshavsky in 1986, suggested that a protein’s lifespan is correlated with the type of amino acid found at its N-terminal end (Varshavsky et al. 2000). Those proteins with a serine residue at their N-terminal end will have a lifespan of more than twenty hours, but those possessing an aspartate residue will have less than three minutes (Varshavsky et al. 2000).

The PEST sequence is a group of eight amino acid residues rich in proline, glutamic acid, serine and threonine (Rogers et al. 1986). Transcription factors with the PEST sequence have short half-lives where its removal immediately increases their survival. This is shown by the transcription factor Gcn4p that contains a normal half-life of five minutes, but the removal of the PEST sequence increases its half-life to fifty minutes.

Lastly, hydrophobic amino acids are normally found within a protein’s hydrophobic core but if the protein becomes abnormal and/or mutated, these hydrophobic residues can become misplaced due to the protein’s partially unfolded state and may be subject to ubiquitylation (Glickman and Ciechanover 2002).

Proteins can be degraded by a number of pathways (Shi et al. 2007):

1) Lysosomal
2) Calcium-dependent
3) Ubiquitin-proteasome
It is important to note that it is possible for a protein to be degraded by more than one mechanism at any one time, and that different pathways can concurrently interact with each other during this process (Hasselgren 1999).

The non-lysosomal, ubiquitin-proteasome pathway plays an important role in intracellular proteolysis. A review by Ciechanover thoroughly describes the importance of proteolysis in mediating a diverse range of regulatory function involved in cell cycling and division; growth and differentiation; activation and silencing of transcription (particularly the silencing of nuclear factor kappa-beta (NFκβ) as will be examined in our study); apoptosis; immune and inflammatory functions; signal transduction; receptor mediated endocytosis; protein sorting and quality control; and metabolic pathways (Ciechanover 2005).

There are two types of ubiquitylation; (1) monoubiquitylation and (2) polyubiquitylation. Monoubiquitylation is a shuttling mechanism, directing target proteins to other subcellular regions such as the nuclear pore complex (NPC), causing the lysosomic degradation of target proteins (Ciechanover 2005). Polyubiquitylation on the other hand, is a multi enzymatic process that forms an isopeptide bond between an α-carboxyl group and a C-terminal glycine that acts as a signal for protein proteolysis within the proteasome (Bebington et al. 2001). Polyubiquitination through Lys-63-linked (K63) chains controls protein kinase activation, DNA repair as well as vesicle trafficking and plays an important regulatory function in NF-κβ signal transduction (Sun and Chen 2004), which will be discussed in detail on Section 1.8.

Krappmann and colleagues suggest that the ubiquitin system controls the NF-κβ pathway in many different ways. One of the pathways includes the phosphorylation of the inhibitor of kappa beta kinase complex (Punnonen et al. 1980) priming the inhibitory molecule for lysine Lys-48-linked (K48) polyubiquitination and degradation by the 26S proteasome (Krappmann and Scheidereit 2005).
As the NFκβ pathway is a known transcriber of survival factors in endometriosis (Gonzalez-Ramos et al., 2007), whereby ubiquitin plays an intermediary role through kinase activation, (Blackwell and Christman 1997), we hypothesize that an increase in ubiquitin expression may cause ectopic endometrial cell survival at ectopic locations rather than degradation, by activating an intermediary kinase mediated in the NFκβ pathway. One such kinase is IKK which is tagged directly by ubiquitin, aiding kinase degradation within the proteasome (Tansey 2004). IKK degradation allows the release of NFκβ (an indirect consequence of ubiquitination) to translocate into the nucleus by phosphorylating inhibitor of kappa beta (Ikβ) in the cytoplasm (Sun and Chen 2004) and initiate the transcription of survival factors such as baculoviral inhibitor of apoptosis protein repeat containing 2 (cIAP2), B-cell leukaemia/lymphoma – X longer transcript (BCL-Xₐ), B-cell leukaemia/lymphoma 2 related protein A1 (BFL1), Fas associated death domain like interleukin 1 β-converting enzyme (FLICE) inhibitory protein (FLIP), IL-8 and TNF-α (Nakanishi and Toi 2005). In the present study, any significant difference in levels of ubiquitin, IKK, NFκβ and the proteasome proteins within endometriosis and control endometrium, may reveal the possible involvement of the ubiquitin-proteasome and NFκβ system in the pathogenesis of endometriosis. In particular, since no study has specifically looked at the role of NFκβ in baboons with endometriosis, we wanted to determine if the protein played a role in this animal model.

1.5.2 Ubiquitin-proteasome pathway
Before target proteins are sent to the proteasome for degradation, they first must be recognised and tagged by the ubiquitin system and this is accomplished with the aid of the E1, enzyme-2 (E2), ubiquitin carrier protein/conjugating enzyme and enzyme-3 (E3), ubiquitin protein ligase enzyme (Figure 1.4).

E1 enzymes are the ubiquitin activating enzymes that are responsible for modifying ubiquitin proteins, so that its terminal glycine residue may bind to the terminal lysine residue of substrate protein. The E2 enzyme, also known as the ubiquitin conjugating
enzyme, catalyses the attachment of ubiquitin and the substrate protein. The final E3 enzyme is the ubiquitin ligase that assists in the recognition of substrate proteins and works alongside the E2 enzymes. Once the target proteins are recognised and tagged, they are sent to the proteasome.

Figure 1.4: The ubiquitin-proteasome mediated pathway. (Coux). “Regulation of the ubiquitin-proteasome system.”

1.5.3 Proteasome structure and function

The proteasome is a major non-lysosomal pathway, expressed both in the nucleus and the cytoplasm of eukaryotic cells that is responsible for the degradation of misfolded and short-lived proteins, as well as the regulation of cell proliferation, inflammation (Meiners et al. 2002), cell cycling and survival. Studies by Hershko, Ciechanover and Rose in the late
70’s and early 80’s led to the discovery of the system, which subsequently led to their shared 2004 Nobel Prize in Chemistry (Kresge et al. 2006).

The proteasome is usually called the 26S proteasome, which is made up of two sub complexes. An inner 20S proteasome (20S) proteolytic core of identical β subunits and two outer 19S proteasome (19S) proteolytically inactive regulatory α subunits, at both ends of the 20S particle (Davy et al. 2001) (Figure 1.5). The 19S component is responsible for substrate recognition as well as the recognition of short and long-lived proteins (Schwartz and Ciechanover 1999), whilst the 20S component is involved in the intracellular breakdown of non-ubiquitylated segments. The ‘S’ refers to the unit of measure called the sedimentation coefficient, or the Svedberg coefficient.

Together, the outer and inner rings make up the ‘stacked-ring’ complex, each containing seven proteins. The ‘stacked-ring,’ structure was first solved using electron microscopy in the 80’s (Kopp et al. 1986) followed by the determination of its core component by X-ray crystallography in 1994 (Lowe et al. 1995). Presently, almost all genes coding the proteasomal component are known; however, what is yet to be determined, is their specific biological roles (Tanahashi et al. 1999).

Although the α and β subunits have similar protein structures, the α subunit contains an extra helix that is thought to be the first part of the proteasome that recognises the degradation sequences on the target protein (Bogyo et al. 1997) (Figure 1.5). The α subunit acts as a ‘gate,’ by controlling the entry of polyubiquitin tagged proteins and is representative of the number of 20S proteasomes present in the cell, as the 20S subunit is not usually found in isolation (Bardag-Gorce et al. 1999).

Recognition of proteins by the proteasome is a multi-step process requiring energy. The protein must first be marked by ubiquitin, polyubiquitinated and subsequently degraded by the 20S proteolytic core. Any step affected in the pathway may have dire consequences for
the cell as it may prevent cell growth, development and proliferation, leading to programmed cell death (Tanahashi et al. 1999). For example, substrates such as transcription factors and inhibitory molecules may not be degraded causing a dysregulation in the cell cycle. Moreover, blocking proteasome activity may stabilize inhibitory proteins contributing to cell growth arrest and apoptosis (Chauhan et al. 2005).

Once the target protein is recognised, it is guided into the β subunits’ proteolytic core and broken down into smaller peptides. To make this possible, ATP molecules are hydrolysed before substrate translocation into the proteolytic, ‘barrel-like’ 20S chamber, to allow proteins to pass in a partially unfolded form.

**Figure 1.5:** Schematic view of the 26S proteasome (26S), which is made up of two outer 19S subunits that recognize polyubiquitinated target proteins and the inner catalytic 20S subunit that allows protein degradation (Adams 2004).
This is necessary as the core chamber is too narrow to allow the passage of proteins in their folded conformation. These peptides can be re-used by the cell to build other proteins, or be directed to the endoplasmic reticulum and subsequently to the immune system by the major histocompatibility complex-1 (MHC-1) (Glickman and Ciechanover 2002).

Only 20% of the proteasome activity is necessary for maintaining normal ubiquitin-proteasome function (Lindsten and Dantuma 2003). A decrease in proteasomal expression for example, may not necessarily indicate an abnormal proteolytic activity, as only a small percentage is needed for functional proteolytic integrity to remain.

**1.6 The Ubiquitin pathway and endometriosis**

*1.6.1 Why look at the ubiquitin system in endometriosis?*

As established earlier, the ubiquitin-proteasome system is a major protein degradation mechanism in the non-lysosomal pathway that has been linked to apoptosis (Meiners *et al.* 2002). Studies by Bebington and colleagues have shown that ubiquitin and its cross reactive protein are found within the luminal epithelium of the human endometrium as well as in decidual cells of the placenta during early pregnancy (Bebington *et al.* 1999). This suggests that ubiquitylation is participating in the differentiation of endometrial cells and possibly also in tissue remodelling and development, especially as the endometrium regenerates in a cyclic manner during the female reproductive cycle. Bebington and colleagues also show that baboon uterine tissues have similar distribution patterns for ubiquitin and its cross reactive protein, suggesting that the baboon may be an adequate model for the study of endometriosis (Bebington *et al.* 1999).

The ubiquitin protein bears a similar sequence homology to the B-cell leukemia/lymphoma-2 (Bcl-2) gene, which has been extensively studied and shown to prolong the life of a cell by preventing apoptosis (Schwartzman and Cidlowski 1993; Watanabe *et al.* 1997). Thus an up-regulation of ubiquitin and its cross reactive protein during the menstrual cycle may provide a protective mechanism for endometrial cells shed
within the effluent, assisting the cells to survive their journey to ectopic locations and invade these areas to form endometriotic implants.

1.7 Apoptosis

1.7.1 History and detection

The first group to describe apoptosis, or 'programmed cell death,' were Kerr and colleagues who saw a process in cells that first involved a nuclear and cytoplasmic condensation and a later division into discrete membrane bound bodies with organelles (Kerr et al. 1972). They gave the ancient Greek name apoptosis to this discrete membrane body formation, for it mimics the "falling off" of petals or leaves (Kerr et al. 1972). Apoptosis is different to non-programmed cell death such as necrosis, for it is genetically programmed and is not induced by an injurious stimulus. Programmed cell death has been described as a selective, multi-step, physiological and pathological process that controls cell shape, numbers and size, by balancing the levels of pro-apoptotic and anti-apoptotic family gene members, ensuring tissue homeostasis (Darzynkiewicz et al. 1997; Tao et al. 1998; Willingham 1999). Under an electron microscope, apoptotic cells have condensed nuclear chromatin, compact cytoplasmic organelles, and protein protuberances on their surface, as well as DNA fragmentation (Darzynkiewicz et al. 1997). In addition, cytoskeletal disruption, cell shrinkage and membrane blebbing can be observed (Hu 2003). These morphological changes can be examined under a light microscope by using the terminal deoxynucleotidyl transferase (TUNEL) technique. TUNEL aids in the topographical location and quantitation of apoptotic cells within cell populations, by incorporating a terminal deoxynucleotidyl transferase with biotinylated deoxyuridine at the 3' -OH ends of the DNA strand breaks (Gavrieli et al. 1992). Currently, no single assay exists that detects apoptosis with complete specificity and sensitivity. The TUNEL technique must be used in conjunction with other classical methods such as haematoxylin and eosin (H&E) staining, that detects nuclear shape changes during the early stages of apoptosis, because positive strand break detection may overestimate the true occurrence of cell death within a given cell population. A positive staining for DNA strand breaks for example may not correlate
with nuclear segmentation or may be detected during the late lytic stage of apoptosis, where most cells are no longer viable.

1.7.2 Apoptosis in the normal endometrium

During the menstrual cycle, the uterine endometrium of healthy, fertile women responds to changing oestrogen and progesterone levels by cyclic tissue breakdown and regeneration processes. Apoptosis is one of the many factors involved in this cyclic shedding, as known apoptosis inducing receptors tumour necrosis factor (TNF) receptor superfamily member (Fas) and TNF are continuously expressed in the eutopic endometrium (Agic et al., 2009), although a large number of cells remain viable at menstruation.

Furthermore, it has been shown that apoptosis may be hormonally regulated by the presence of local receptors for 17β-oestradiol and progesterone and proteins such as Bcl-2, that act on essential factors required for the regeneration of the endometrium after events such as menstrual shedding (Dahmoun et al. 1999).

Using antibodies to detect the 17β-oestradiol receptor-alpha (ER-α) and progesterone receptor (PR), and an in situ end labelling method for apoptotic cells, Dahmoun and colleagues showed that glandular and stromal cells react differently to changes in hormone receptor levels. Their study showed that when the proliferative activity of the glandular epithelium was low, there was an associated increase in the number of apoptotic cells. Moreover, these changes seemed to occur when there were decreasing protein levels for ER and PR.

In contrast, there is a late onset of apoptosis in the stromal cells caused by the presence of local hormones that regulate ER and PR, that prolong their steroidal effect so that stromal cells are given time to renew and repair the shed endometrium (Dahmoun et al. 1999).
Evidence that changing protein concentration of the anti-apoptotic marker, Bcl-2, can control apoptosis is shown by a study by Tao and co-workers (Tao et al. 1998). Their study shows that Bcl-2 expression predominates during the proliferative phase of the menstrual cycle, and that B-cell lymphoma 2 family-associated X protein (Bax) expression, a pro-apoptotic protein, increased during the secretory phase (Tao et al. 1998). This finding shows that endometrial cell integrity and survival relies on the final concentration of pro-apoptotic and anti-apoptotic proteins such as Bax and Bcl-2.

The above studies by Dahmoun and Tao show that proteins such as Bcl-2 can hormonally regulate apoptosis and are differentially expressed within the glandular and stromal cells. This is of great interest as Bcl-2 has a similar structural homology to ubiquitin, and any changes caused by this protein may also be comparable to changes induced by ubiquitin.

Although general consensus supports apoptosis to be a naturally occurring mechanism in the human endometrium, conflicting data as to the role that Bcl-2 plays in the regulation of this process is more in contention. Various studies support the idea that Bcl-2 up-regulation has anti-apoptotic properties (Tao et al. 1998) whilst others claim that no significant correlations exist between Bcl-2 and the prevention of apoptosis (Watanabe et al. 1997). The same mechanism may occur with ubiquitin, although our previous study showed that ubiquitin was likely to have anti-apoptotic properties and may take part in ectopic cell survival.

### 1.7.3 Apoptosis in endometriosis

Apoptotic cells within the eutopic and ectopic endometrium of patients with endometriosis were shown to be lower than in control patients (Gebel et al. 1998; Taniguchi et al., 2011) and to have a decreased ability to differentiate (Beliard et al. 2004). This provides an explanation as to why misplaced cells in women with endometriosis are able to thrive, compared to healthy controls for greater resistance to apoptotic stimuli may result in increased cell survival. However, the authors did not find significant differences in
apoptotic activity during the proliferative and secretory phases of the menstrual cycle and were not able to exhibit that differential apoptosis may exist within stromal and glandular cells, which is in contrast to the study by Dahmoun (Dahmoun et al. 1999).

Normally, epithelial cells exhibit apoptotic bodies at late secretory phase, as viewed by electron microscopy (Otsuki et al., 1994). However, this mechanism is impaired in women with endometriosis, whereby the cyclic nature of apoptosis during the menstrual cycle is impaired, resulting in endometrial cells with an increased capacity for survival, particularly those from the early proliferative, late secretory and menstrual phase (Dmowsk et al. 2001).

In addition, women with endometriosis have peritoneal fluid that is abundant in FasL, causing immune cells to exhibit FasL on their surface and thus impairing their scavenger function (Garcia-Velasco et al. 2002). This allows refluxed cells to survive at their new ectopic location, as they are no longer recognized by the immune cells and are disposed of accordingly.

Further investigation of apoptosis and cellular remodelling can also be conducted on ectopic areas such as the mesothelial monolayer of the peritoneum. Weusten and colleagues believe that cellular remodelling at these ectopic locations can be seen with markers for cytokeratin, and can be induced by menstrual effluent paracrine factors that encourages basement membrane exposure and eventual endometrial implant formation (Demir Weusten et al. 2000). Cytokeratin reorganisation can be seen as changes in mesothelial cell morphology, as typically polygonal cells gain an elongated spindle appearance (Demir Weusten et al. 2000).
1.8 Proteins in the Ubiquitin-NF-κβ pathway

1.8.1 NFκβ structure and function

NFκβ is a transcription factor found in simple and complex organisms, such as invertebrates and mammals. Organisms such as sea anemones (Putnam et al. 2007), use NFκβ as an inflammatory response to various stressful stimuli such as cytokines (Middleton et al. 2000; Putnam et al. 2007), free radicals (Muller et al. 1997), ultraviolet radiation (Tanaka et al. 2005), oxidised low density lipoprotein (LDL) (Cominacini et al. 2000), and bacterial (Zhang et al. 1999) and viral antigens (Yoshida et al. 2001). The NFκβ pathway plays an important role in normal immune regulatory response to infection but its deregulation is seen in conditions such as cancer (Shen and Lentsch 2004), and autoimmune diseases such as diabetes (Mollah et al. 2008).

The NFκβ protein complex was first discovered by David Baltimore, a 1975 Nobel Prize winner in Physiology or Medicine (Foundation 1976) through its interaction with sequences on the immunoglobulin light-chain enhancer in B cells. Presently, NFκβ is grouped within the family of NFκβ/Rel proteins, for its structural similarity to the retroviral oncoprotein v-Rel.

There are five main groups in the mammalian NFκβ family:

1) NFκβ1 also known as p50
2) NFκβ2 also known as p52
3) RelA (otherwise known as p65 or RELA: v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa)
4) RelB (RELB: v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa)
5) c – Rel (c-v-rel reticuloendotheliosis viral oncogene homolog)
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The first two groups \( \text{NF}_{\kappa\beta}1 \) (p50) and \( \text{NF}_{\kappa\beta}2 \) (p52), are mature products of their larger precursors nuclear factor kappa beta 1 precursor (p105) and nuclear factor kappa beta 2 precursor (p100) respectively, which involves the ubiquitin-proteasome mediated degradation of C-terminal end ankyrin repeats, which are sequences responsible for protein-protein interactions.

\( \text{NF}_{\kappa\beta} \) is known as a ‘first responder’ protein, as it exists in an inactive state in the absence of harmful stimuli. However, upon stressful cellular insult, such as those described earlier, or upon TNF-\( \alpha \) activation, causes \( \text{NF}_{\kappa\beta} \) to rapidly respond to prevent extensive cellular damage. \( \text{NF}_{\kappa\beta} \) causes cell survival by activating anti-apoptotic proteins of the B-cell lymphoma 2 family (BCL2), such as BCL-\( \text{X}_L \) and BFL1, or by blocking the activation of caspases such as the inhibitor of apoptosis 1 and 2, (IAP1 and IAP2) and the X-linked inhibitor of apoptosis protein (XIAP) (Nakanishi and Toi 2005).

There are two main \( \text{NF}_{\kappa\beta} \) activation pathways (Figure 1.6):

1) The classical, canonical pathway
2) Non-canonical pathway

The classical, canonical pathway begins with TNF-\( \alpha \) and/or LPS activation of the IKK complex I-kappa-B-kinase-alpha, I-kappa-B-kinase-beta and I-kappa-B-kinase-gamma (IKK\( \alpha \), IKK\( \beta \) and IKK\( \gamma \) respectively), causing the phosphorylation of IKK inhibitory molecules for ubiquitin-proteasome mediated degradation (Figure 1.5). The non-canonical pathway on the other hand, is activated by cytokines such as the B cell-activating factor from the tumour necrosis factor family (BAFF) and CD40L, and is regulated by NF-\( \kappa\beta \) inducing kinase (NIK) and IKK\( \alpha \), (not IKK\( \beta \) and IKK\( \gamma \)), to cause NF-\( \kappa\beta \) p100 subunit degradation to p52 (Ramakrishnan et al. 2004).
In the absence of an external, usually harmful stimulus, NFκβ remains sequestered in the cytoplasm in its inactivated form, by the inhibitor of kappa B (IκB), whose multiple ankyrin repeat domain prevents the exposure of NFκβ’s nuclear localisation signal (NLS).

The IκB protein family consists of I-kappa-B-alpha (IκBα), I-kappa-B-beta (IκBβ), I-kappa-B-gamma (IκBγ) and B-cell CLL/lymphoma-3 (Bcl-3). This study focuses on the extensively studied IκBα inhibitor and its role in mediating NFκβ activation. IκB proteins have a regulatory N-terminal domain, several ankyrin repeats and a degradation PEST sequence at its C-terminal end.

Signal transduction of NFκβ originates outside the cell, causing the Iκβ kinase to phosphorylate Serine 32 and/or Serine 36 located on the Iκβα regulatory domain, after TNF-α agonist stimulation (Yamauchi et al. 2004).
Figure 1.6: Schematic view of the inter-relationships between the proteins in the ubiquitin- NFκβ pathway.
The various agonists mentioned earlier, can initiate the phosphorylation of Iκβα by the IKK complex, providing the signal for Iκβα ubiquitin-proteasome mediated degradation, as well as the consequent release of NF-κβ. The liberation of NFκβ allows the protein to translocate into the nucleus and bind to DNA sequences containing the kappa-B motif, to initiate either an inflammatory, cell survival or proliferative response, depending on a cell’s physiological requirements and upon a specific stimulus. These motifs include the 5’-GGGRNNYYCC-3’ and 5’-HGGARNYYCC-3’, whereby H = A, C or T; R = purine bases A or G and Y = pyrimidine bases C or T (Parry and Mackman 1994).

The NFκβ pathway is an interesting system for it is able to cause an auto feedback loop by switching the expression of its own repressor, Iκβα. Thus at any given time, NFκβ can either encourage or control protein expression, as long as DNA binding sites for NFκβ exists in the nucleus. This mechanism plays an important part in the survival of viruses and their pathogenicity. Viruses can either be in their latent, inactivated state, or their pathogenic, activated form, depending on the balance between Iκβα and NFκβ.

As previously mentioned NFκβ controls genes responsible for cell proliferation and survival. However, under uncontrolled conditions, as seen in cancerous cells, NFκβ is said to be ‘constitutively active,’ where cell proliferating genes and survival genes are continuously turned on. An aberrant regulation of this ‘switch’ can contribute to ectopic cell survival in endometriosis (Lousse et al. 2008).

Several studies involving NF-κβ include the investigation of its mRNA transcripts in the normal endometrial tissues of women (King et al. 2001) mobility shift assays to determine its constitutive activity (Gonzalez-Ramos et al. 2007) and its activation within macrophages collected from the peritoneal fluid of women with and without endometriosis (Lousse et al. 2008). Peritoneal macrophages in patients with endometriosis have increased levels of NFκβ activation (Lousse et al. 2008) and are known to modulate the
inflammatory and adherence mechanisms commonly associated with the condition (Dunselman et al. 2001).

Mutations in NFκβ transcription factors and/or their inhibitors (i.e. Iκβ), or the excessive secretion of TNF-α found in tumours (Wilson 2008) and ectopic endometrial lesions (Lee et al. 2008), can cause the aberrant regulation of NFκβ, and subsequently an uncontrolled cell growth. Thus therapeutic intervention of any components within the NFκβ pathway may prove an effective treatment for conditions such as cancer and endometriosis.

A number of inhibitor studies involving NFκβ and endometriosis include the modulatory role of progesterone and its compound derivatives, dienogest and danazol on NF-κβ mediated activation in endometriotic stromal cells (Horie et al. 2005). Other compounds with promising therapeutic effects include human chorionic gonadotrophin (hCG) hormone, as it prevents the phosphorylation and degradation of Iκβα, suppressing NF-κβ nuclear translocation (Huber et al. 2007). In addition, BAY 11-7085, a soluble NF-κβ inhibitor, has been shown to down-regulate the anti-apoptotic proteins Bcl-2 and Bcl-XL, increasing apoptotic proteins caspase-3, as well as cleaved caspase 8 and 9, in stromal cells isolated from women with endometriotic cysts (Nasu et al. 2007). Moreover, gonadotrophin-releasing hormone agonist (GnRHa) (Sakamoto et al., 2003) and thalidomide (Tha1) (Yagyu et al. 2005) have been shown to attenuate IL-8 expression by decreasing TNF-α mediated NF-κβ activation, in women with the condition. Whilst a NSAID with NF-κβ inhibitory capabilities, Sulindac, reduces the inflammatory reaction associated with endometriosis by preventing the NF-κβ mediated gene expression of the chemokine regulated on activation normal T cell expressed and secreted (RANTES) protein (Wieser et al. 2005).

Interestingly, an NF-κβ inhibitor, pyrrolidine dithiocarbonate (PDTC) and a proteasome inhibitor called bortezomib, has recently been used to decrease the severity of peritoneal
endometriosis induced in rats (Celik et al. 2008). Moreover, NF-κβ inhibitors initiate apoptosis and a reduction in the inter-cellular adhesion molecule 1 (ICAM-1) on induced disease, as measured by diminishing changes in lesion fluorescence and consequently its size in luminescent mice models (Gonzalez-Ramos et al. 2008).

NFκβ controls genes involved in inflammation, commonly found in the innate and adaptive immune response. This is the main reason why endometriosis is often mistaken for another inflammatory related condition called inflammatory bowel disease (IBD) and shares similar inflammatory characteristics with arthritis (Lazzerini et al. 2007), and asthma (Zhou et al. 2008).

The investigation of the ubiquitin mediated NF-κβ pathway in endometriosis is a relatively new field. In endometriosis, NF-κβ activation is a key component of the inflammatory reaction that increases the transcription of TNF-α to provide a feedback mechanism for further NF-κβ activation (Sakamoto et al. 2003). NFκβ can also induce pro-inflammatory cytokine transcription and reactive oxygen species (ROS) generation (Yamauchi et al. 2004), as well as adhesion molecule, inflammatory, stress and immune-system receptor protein transcription (Schwartz and Ciechanover 1999). One transcription factor in particular involves the activation of NF-κβ as a response to ROS development from iron exposure (Seldon et al. 2007), where elevated iron levels may overwhelm detoxification systems allowing the continued growth of endometriotic cells (Yamaguchi et al. 2008). In addition, the ability of miR-199a to target IKBKB in NF-κβ signalling and its pro-inflammatory action in ovarian cancer (Chen et al. 2008b) is a mechanism that may be relevant to endometriosis.

Another possible pathway involved in endometriosis development and its associated inflammatory response is an IL-1β mediated NF-κβ nuclear translocation and activation of the macrophage migration inhibitory factor (MIF), a known mitogenic and pro-inflammatory protein (Veillat et al. 2009). The same study also determined that curcumin,
an NF-κβ inhibitor is able to inhibit this process at two potential regulatory sites (Veillat et al. 2009).

Thus NFκβ can modulate the inflammatory and adherence mechanisms commonly seen in endometriosis.

1.8.2 IKK structure and function
IKK is located upstream of the NFκβ signalling cascade that enables the transcription of the NFκβ complex by phosphorylating its inhibitor Iκβα, which acts as an initiator of lysine-48-linked polyubiquitination (Sotnikova et al. 2002) and its subsequent 26S proteasome degradation (Krappmann and Scheidereit 2005). Iκβα usually masks the NLS on NFκβ, to prevent its translocation into the nucleus. Thus the phosphorylation of Iκβα allows its dissociation from NFκβ to cause the transcription of NFκβ, providing a way for the ubiquitin-proteasome system to control the NF-κβ pathway.

The IKK complex is made up of three subunits namely:

1) IKKα
2) IKKβ
3) IKKγ

There are many synonyms for the catalytically active IKKα, including the conserved helix-loop-helix ubiquituous kinase (CHUK), the inhibitor of kappa light polypeptide gene enhancer in B cells, kinase of alpha (IκBKα), I-kappa-B kinase 1 (IKK1), nuclear factor of kappa light chain gene enhancer in B cells inhibitor, kinase of alpha (NFκBIKα).

The second kinase IKKβ, also has several names such as the nuclear factor of kappa light chain gene enhancer in B cells inhibitor, kinase of beta (NFκBIKβ), the I-kappa-B kinase 2
(IKK2) and the inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKBKB). IKKβ like IKKα contains a catalytically active function. IKKα has an identical function to IKKβ as it can mediate Iκβ phosphorylation and its subsequent NF-κβ activation (Sun and Chen 2004).

The third subunit IKKγ, commonly referred to as the nuclear factor kappa beta essential modifier (NEMO) or the inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma (IKBKG), does not have a catalytically active component and instead serves a regulatory function.

NF-κβ is normally associated with Iκβ and is sequestered in unstimulated cells. Agonists such as TNF-α, Interleukin-1 beta (IL-1β) and phorbol ester (PMA), stimulate NF-κβ by initiating Iκβ phosphorylation through the IKK complex (Sun and Chen 2004). Thus the down-regulation of IKKα and IKKβ, that make up the catalytic complex, should be associated with a decreased level of Iκβ phosphorylation (Sakurai et al. 2003). For example, when the catalytic activity of IKKβ is inhibited, NF-κβ is unable to respond upon TNF-α stimulation (Karin et al. 2004).

IKK phosphorylates Iκβα at residues Ser 32 and/or Ser 36, which initiates Iκβα polyubiquitination at Lys 21 and Lys 22 and its subsequent proteasomal degradation. Iκβα’s are associated with NFκβ within the cytoplasm where its proteasomal degradation provides the trigger for NFκβ nuclear translocation and the activation of survival factors.

To detect the inflammatory response involving TNF-α in endometriosis (Goldberg 2005) through NF-κβ activation, there should be an associated p-Iκβ observed (Sakamoto et al. 2003). The levels of phosphorylated Ser32/Ser36 residue/s on Iκβα will be investigated in this study.
1.8.3 TNF-α structure and function

TNF is a 17kDa protein made up of 212 amino acids (Tang et al. 1996) that forms a stable transmembrane trimeric structure, made up of two antiparallel β sheets and an antiparallel β strand. TNF was first identified as a cytokine secreted by endotoxin-activated macrophages capable of inducing tumour necrosis (Carswell et al. 1975). The TNF cytokine family includes TNF-α and TNF-β. TNF-β was initially called lymphotoxin (LT), however cDNA cloning revealed the structural homology between TNF-β and LT that subsequently lead to its present TNF-β nomenclature (Pennica et al. 1984).

TNF causes an inflammatory response to stressful stimuli, such as the exposure to LPS found on the outer membrane of pathogenic (Gram-negative) bacteria, and interleukin-1 (IL-1). TNF can be found in many different cell types such as macrophages (Torrado et al. 2007), mast cells (Old 1985), neuronal tissues (Hofman and Hinton 1992), and liver cells (Onda et al. 2000), taking part in a variety of functions such as phagocytosis, inflammation and chemotaxis. Generally, an increase in TNF concentration causes an inflammatory response characterized by swelling, heat and pain and is the body’s immune reaction to harmful stimuli.

TNF-α has been implicated in a variety of abnormal states such as insulin resistance and diabetes (Zinman et al. 1999), obesity (Rosmond et al. 2001), hyperandrogenism (Escobar-Morreale et al. 2001), septic shock (de Groof et al. 2002), cerebral malaria (Knight et al. 1999), alopecia areata (Galbraith and Pandey 1995), rheumatoid arthritis (Mulcahy et al. 1996), osteoporosis and osteopenia (Ota et al. 2000), asthma (Winchester et al. 2000), inflammatory bowel disease (Fowler et al. 2005), hepatitis B (Kim et al. 2005), cystic fibrosis (Buranawuti et al. 2007), tuberculosis (Stein et al. 2005), and psoriasis (Boyman et al. 2004).

There are two main TNF receptors, TNF Receptor 1 and 2 (TNF-R1 and TNF-R2). TNF-R1 is expressed in many different cell types and can be activated by the soluble or
membrane bound TNF, while TNF-R2 responds to the membrane bound TNF only and is found predominantly in cells derived from the immune system (Daniel and Wilson 2008).

TNF activates TNF-R that recruits adaptor proteins downstream such as Fas-Associated death domain (FADD), TNF receptor-associated death domain (TRADD), receptor interacting protein (RIP) and tumour necrosis factor receptor associated factor 2 (TRAF2), to cause the transcription of three main pathways to form either an inflammatory, cell survival or apoptotic response. These pathways include:

1) Mitogen activated protein kinase (MAPK)
2) Caspase-8
3) NF-κβ

The MAPK pathway is involved in apoptosis, differentiation and mitosis by utilising the c-Jun N-terminal kinases (JNK) that cause cytokine production through the transcription of activation protein-1 (AP-1) in the nucleus (Oltmanns et al. 2003).

The binding of TRADD to FADD causes the recruitment of the initiator caspase-8 that subsequently cleaves inactive effector caspases to cause apoptosis.

Lastly, the NF-κβ pathway as described in Section 1.8, is involved in cell survival and transcription (Figure 1.5).

In the human endometrium, TNF-α is one of several mediators regulated by NF-κβ pathway and is a stimulator of cell death, whose apoptotic effect can be reversed by NFκβ, allowing cell survival (Hu 2003). TNF-α is also a known activator of other cytokines that are commonly up regulated in the peritoneal fluid of patients with endometriosis (Scholl et al., 2009). This inflammatory response has many regulatory functions ranging from
endocrine, paracrine and autocrine control (Brieland et al. 2001). TNF-α activation of NF-κβ can protect cells against apoptosis, consequently TNF-α antagonists can inhibit NF-κβ activation, preventing ectopic cell survival in endometriosis (Nakanishi and Toi 2005).

Ovarian steroids regulate TNF-α levels in glandular and stromal cells, increasing TNF-α during the proliferative phase, and reaching its peak at late secretory phase (Tabibzadeh et al. 1995; Tanahashi et al. 1999). Thus TNF-α is important in maintaining endometrial cell homeostasis, growth or inhibition (Iwabe et al. 2000).

TNF-α and its related proteins have varying effects on endometrial glandular and stromal cell attachment in endometriosis. A dose-dependent increase in endometrial stromal cell attachment to a sub-confluent culture of peritoneal mesothelial cells (PMC) treated with TNF-α exists (Zhang et al. 1993), whilst treatment with TNF-α, IL-6 and IL-8 prevents glandular cells from attaching to PMC (Debrock et al. 2006). It seems that TNF-α may prevent or facilitate early endometriotic tissue development depending on the tissue type. In addition, the invasive/proliferative effect of TNF-α was supported by other studies utilizing cultured endometrial stromal cells (Iwabe et al. 2000) and eutopic/ectopic stromal cells from patients with endometriosis (Braun et al. 2002).

TNF-α encourages the proliferation of endometriotic stromal cells by causing IL-8 gene and protein expression (Sakamoto et al. 2003) due to NFκβ activity (Yagyu et al. 2005). Cell culture studies by Iwabe and colleagues show a dose dependent induction of IL-8 with TNF-α, along with endometrial stromal cell proliferation (Iwabe et al. 2000). IL-8 plays a key role in endometrial cell proliferation via an autocrine mechanism, exerting its proliferative effect on both normal and endometriotic tissues. Endometriotic tissues are known to produce IL-8, thus any growth regulatory mechanism this protein possesses may be attributed to an autocrine and paracrine function.
TNF inhibition is useful for conditions that cause an aberrant inflammatory response, whereby the use of the TNF-receptor-IgG fusion protein etanercept (brand name Enbrel) for example, can have therapeutic potential for the treatment of endometriosis in the baboon (Barrier et al. 2004).

Other TNF inhibitors include infliximab (brand name Remicade) and the third generation adalimumab (brand name Humira), which are compounds that can down-regulate the TNF inflammatory response. Infliximab is a murine and human chimeric construct, whilst adalimumab is a human monoclonal antibody. Moreover, the TNF-α inhibitor etanercept is effective at preventing peritoneal fluid cell induced growth, further illustrating TNF-α’s role in disease development (Braun et al. 2002).

A time course analysis of TNF-α shows that the phosphorylation of Iκβ causes Iκβ activation and degradation, which is essential in NF-κβ liberation and transcription (Yamauchi et al. 2004). The phosphorylation of p65 on Ser-536 occurs before Iκβα degradation and its subsequent nuclear translocation and activation of NF-κβ. TNF-α mediated activation leads to Ser-536 phosphorylation on p65 in the cytoplasm and its eventual dephosphorylation in the nucleus (Sakurai et al. 2003).

1.9 Aims of the study

Our previous study of the eutopic endometrium from women undergoing laparoscopy for non-endometrial pathologies such as leiomyomata and benign ovarian cyst, revealed that ubiquitin may play a role in endometrial development, in anticipation for a possible embryo implantation, as maximal ubiquitin immunostaining occurs at day 14 of the menstrual cycle (Ilad et al. 2004). We showed that the expression of ubiquitin during the proliferative phase of control tissues is greater than the secretory phase and is likely to be modulated by increasing oestrogen since there is a rise in oestrogen level in response to FSH during folliculogenesis (Ilad et al. 2004). We interpreted our results as indicating that ubiquitin
may take part in ectopic endometrial cell survival, as the increase in ubiquitin was correlated with a decreased level of apoptosis, although at the time, we did not speculate as to which pathway this was mediated by. For this PhD thesis, we are interested in identifying intermediary proteins within the ubiquitin- NF\(\kappa\)B pathway that may be candidates for cell survival within the eutopic endometrium that by implication may provide an insight to the survival of ectopic endometrial cells in endometriosis. We are also interested in seeing whether a similar pattern of ubiquitin is present within baboons, further verifying whether the baboon animal model adequately mimics the human endometrial milieu.

We will compare the nuclear and cytoplasmic expression of proteins involved in the pathway within glandular and stromal cells during ML phase, to determine their localisation. In particular, we are interested to see if NF\(\kappa\)B is located in the nucleus of endometriotic tissues, providing the possibility that its anti-apoptotic activity is involved in ectopic cell survival.

Our study will investigate the 19S proteasome regulatory subunit, which contains numerous functions including: (1) recognition of polyubiquitinated proteins, (2) opening of the proteolytic chamber (Shi et al. 2007) and unfolding of substrates to adequately fit in the 20S chamber (Ciechanover 2005).

Any changes in proteasomal expression and function between the eutopic and ectopic endometrium may be attributed to several reasons including: (1) a distortion in the proteasome subunit composition, (2) a change in proteasome protein content, (Shi et al. 2007) a decrease in the proteasome regulatory complex and (4) an oxidation of the proteasome cysteine residues (Deruisseau et al. 2005).
We will also investigate the effect of IKK phosphorylated p65 subunit of NFκβ on Ser-536 to determine the possible activation of NFκβ (Sakurai et al. 2003), which may potentially lead to endometrial cell survival.

In addition, as TNF-α is known to induce cell growth in endometriosis, a paradoxical finding as the protein has known apoptotic effects on many different cell types and models, we are interested to see if TNF-α can cause cell survival or death in endometrial cells. In this study we investigate the role of TNF-α induced phosphorylation of p65 on Ser 536 of NF-κβ, using an anti-phospho-p65 antibody.

Molecular techniques on ribonucleic acids (RNA) extracted from endometrial tissues and immunohistochemistry on formalin fixed, paraffin tissues will be used to investigate key components of the ubiquitin- NFκβ pathway in women and baboons.