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- Promotors

Professor Jacques Donnez (UCL)

Doctor Anne Van Langendonckt (UCL)

A mi madre alegre, inteligente, hermosa y valiente

A mi padre idealista, luchador incansable y ejemplo académico

A mi abuela Gina, fuente de amor y alegría

A mi abuelo José Domingo Ramos Albornoz, el coronel honrado, ético y valeroso, nuestro pilar, mejor amigo y abuelo de todos

A mi hermana Daniela, presente y futuro esplendor

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Finalement, je vais me permettre d'écrire quelques mots en espagnol pour remercier ma famille.

Gracias querida familia por toda la ayuda que me han dado desde siempre y durante estos años de ausencia, los quiero mucho y estoy feliz de volver a verlos pronto y poder mostrar con orgullo este trabajo que sin duda es en gran parte gracias a vuestro apoyo.

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Abbreviations

AR	**Androgen receptor**
CI	**Calcium ionophore**
COC	**Combined oral contraceptives**
COX-2	**Cyclooxygenase-2**
E	**Estradiol**
ECM	**Extracellular matrix**
EGF	**Epidermal growth factor**
EMSA	**Electrophoretic mobility shift assay**
ER	**Estrogen receptor**
GnRH	**Gonadotropin-releasing hormone**
GnRH-a	**Gonadotropin-releasing hormone agonists**
HGF	**Hepatocyte growth factor**
ICAM-1	**Intercellular adhesion molecule-1**
IFN-γ	**Interferon-gamma**
IκB	**Inhibitor of NF-κB**
IKK	**IκB kinase**
IL	**Interleukin**
IL-1RI	**IL-1 receptor type 1**
iNOS	**Inducible nitric oxide synthase**
IVF	***In vitro* fertilization**
LIF	**Leukaemia inhibitory factor**
LPS	**Lipopolysaccharide**

MIF	**Macrophage migration inhibitory factor**
MCP-1	**Monocyte chemoattractant protein-1**
MMP	**Matrix metalloproteinase**
Mn-SOD	**Manganese superoxide dismutase**
NF-κB	**Nuclear factor-kappa B**
NIK	**NF-κB inducing kinase**
NSAIDs	**Non-steroidal anti-inflammatory drugs**
OD	**Optical densitometry**
P	**Progesterone**
PCB	**Poly-chlorinated biphenyl**
PPARγ	**Peroxisome proliferator-activated receptor γ**
PR	**Progesterone receptor**
RANTES	**Regulated on activation, normal T cell expressed and secreted**
RNA	**Ribonucleic acid**
ROS	**Reactive oxygen species**
TIMP	**Tissue inhibitor of metalloproteinase**
TNF-α	**Tumor necrosis factor-α**
TPA	**Phorbol, 12-myristate, 13 acetate**
TUNEL	**TdT-mediated biotin-dUTP nick-end labeling**
uPA	**Urokinase type of plasminogen activator**
VEGF	**Vascular endothelial growth factor**

Overview

Endometriosis is one of the most frequently encountered benign diseases in gynecology, being the cause of dysmenorrhea, dyspareunia, chronic pelvic pain and infertility in more than 35% of women of reproductive age. Decreased quality of life may result not only from the symptoms of pelvic pain and infertility, but also from the side effects of various medical and surgical treatments. For women with pain, surgery commonly provides temporary relief, but symptoms recur in up to 75% of women within 2 years, and further surgery is needed in many cases. The most widely accepted hypothesis on the origin of endometriosis is Sampson's theory of retrograde menstruation. According to this theory, endometriosis originates from endometrial cells regurgitated through the Fallopian tubes, which have the ability to survive, adhere, invade tissues, create a blood supply and proliferate outside their eutopic location. Endometriosis is nowadays considered to be a multifactorial and enigmatic disease. Anatomic, genetic, environmental, hormonal, immunologic and oxidative stress factors have been implicated in the establishment, development, maintenance and progression of endometriotic lesions

Local immunity has been found to be altered in peritoneal endometriosis. Abnormal levels of multiple cytokines and chemokines are present in the peritoneal fluid of endometriosis patients, resulting in a proinflammatory local environment. Cytokines, chemokines,

adhesion molecules and/or growth-promoting factors involved in endometriosis-associated inflammatory reaction include interleukin (IL)-1, IL-8, tumor necrosis factor (TNF)-α, regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein (MCP)-1, intercellular adhesion molecule (ICAM)-1, matrix metalloproteinase (MMP)-1 and macrophage migration inhibitory factor (MIF).

The transcription factor nuclear factor-kappaB (NF-κB) plays a key role in the immune and inflammatory response, modulates cell proliferation and apoptosis in many cell types and has also been implicated in adhesion, invasion and angiogenesis. Interest in this molecule in the context of endometriosis has been steadily growing in recent years. To date, there is no direct evidence involving NF-κB activity in the inflammatory response of endometriosis *in vivo*. Furthermore, the effect of NF-κB activation or inactivation on cell proliferation and apoptosis in endometriosis, have not been previously studied. The aim of this study was to investigate the involvement and role of NF-κB in peritoneal endometriosis *in vivo* and its potential as a novel central therapeutic target for the prevention and treatment of endometriosis.

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Chapter I: Introduction

1. Endometriosis

1.1. Definitions

1.1.1. Endometrium and the menstrual cycle

Endometrial physiology

The endometrium is the internal layer of the uterus, composed of epithelial glands and stroma. The endometrium proliferates under the influence of estradiol (E) during the follicular phase (proliferative phase) of the ovulatory cycle (menstrual cycle). During the luteal phase (secretory phase), the endometrium is prepared for implantation by the action of progesterone (P). In case of fertilization, the blastocyst may implant in the endometrium upon its arrival in the uterus. If pregnancy fails to occur, the corpus luteum regresses and levels of P and E fall suddenly (Figure 1). P withdrawal is the physiological signal for menstruation. Progesterone receptor (PR)-positive stromal cells in the superficial zones of the endometrium respond to this decrease and initiate the menstrual cascade. This cascade involves increased production of prostaglandins generating vasoconstriction and hypoxia, greater expression of vascular endothelial growth factor (VEGF) and its receptor, elevated levels of stromal matrix metalloproteinases (MMPs), leukocytic invasion, and perivascular expression of cytokines and MMPs. MMPs have been

identified as the key class of proteinases involved in the initiation of menstruation (Marbaix *et al*, 1996; Brenner *et al*, 2002). During menstruation, the superficial endometrial zones are sloughed off. Menstrual endometrium is eliminated through the uterine cervix and vagina, but retrograde flow of the menstrual slough through the Fallopian tubes occurs in most women (Halme *et al*, 1984).
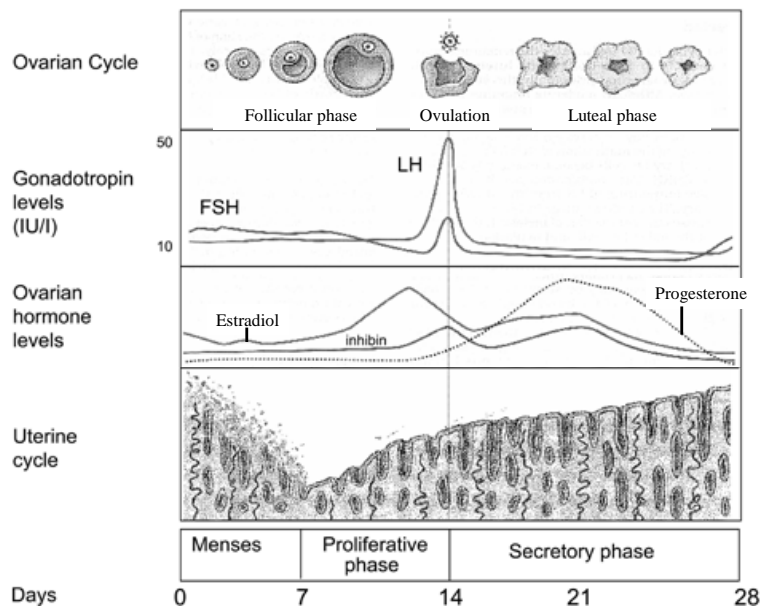
Figure 1: Menstrual cycle. FSH: follicle-stimulating hormone, LH: luteinizing hormone (adapted from Kaplan and Manuck, 2004).

Endometrial histology

Human endometrium has two major components: endometrial glands and stroma (Figure 2). Endometrial glands consist of simple columnar

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epithelium (epithelial cells) forming numerous tubular glands, which are supported by endometrial stroma. Endometrial stroma is composed of a variety of cells (stromal cells), including spindle-shaped connective tissue cells and bone marrow-derived cells. Both glands and stroma undergo extensive changes during the menstrual cycle.

Two layers have been characterized in the endometrium:

a. A superficial layer or stratum functionalis that is sloughed off during menstruation and redevelops with each new cycle.

b. A deep layer or stratum basalis composed of permanent stromal tissue and the blind ends of the uterine glands. The stratum basalis is maintained permanently during the menstrual cycle and provides the stratum functionalis with a cell source.

Endometrial glands have quite a flat form and mitotic cells are often found during the proliferative phase. During the secretory phase, epithelial glands are composed of mature secretory cells and their shape is more tortuous (Figure 2).

During the proliferative phase, 10-15% of cells in the stroma are positive for the leukocyte-common antigen CD45, increasing to 20-25% in the late secretory phase. T cells and macrophages are the major leukocyte populations in the stroma, with T cells most often found in aggregates within the deeper layers of the endometrium, and macrophages scattered throughout the endometrium. The density of

macrophages increases during menstruation (Fernández-Shaw *et al*, 1995).
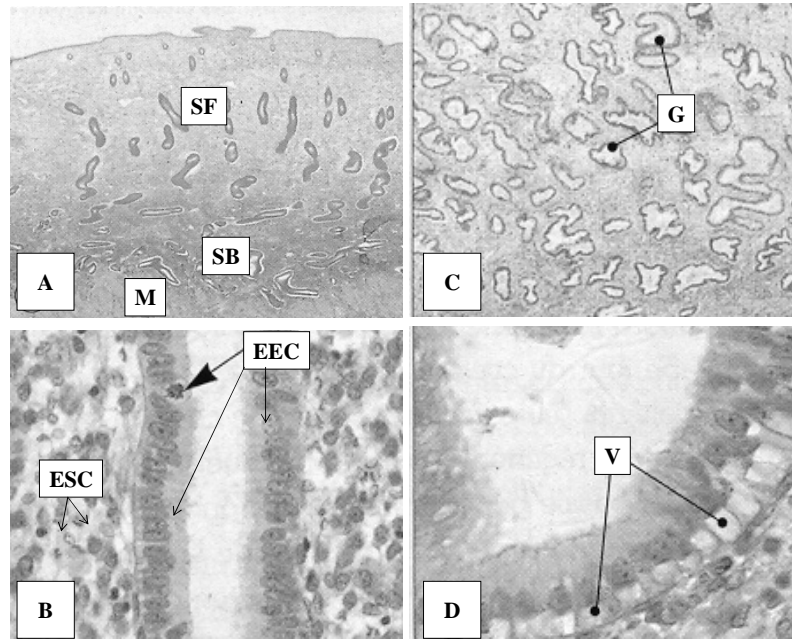
Figure 2: Histology of endometrium. Pictures A and B show endometrial histology during the proliferative phase of the menstrual cycle, and C and D during the secretory phase. SF: stratum functionalis, SB: stratum basalis, M: myometrium, G: endometrial glands, EEC: endometrial epithelial cells, ESC: endometrial stromal cells, V: vacuoles (adapted from Stevens and Lowe, 1997).

Another important component of the endometrial stroma is the vascular network, which also participates in the menstrual cycle. During menstruation, distal vessels are sloughed off, while spiral arteries retract into the stratum basalis and constrict to limit blood loss. During the proliferative phase, spiral arteries extend again as the stratum functionalis redevelops.

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1.1.2. Endometriosis

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterine cavity. The most common locations are the pelvic peritoneum and the ovaries. Extraperitoneal endometriosis also exists, notably in the rectovaginal septum. Indeed, this disorder can be found in all organs, except the spleen (Markham *et al*, 1989; Nisolle and Donnez, 1997).

1.2. Epidemiology

Endometriosis is one of the most frequently encountered benign diseases in gynecology. The prevalence of endometriosis may vary depending on the study. In asymptomatic women undergoing tubal ligation, the prevalence is about 4% (1-7%). In women with primary infertility, the prevalence varies from 9 to 50%, while in women with pelvic pain, it ranges from about 5 to 21%. Around 50% of adolescents with intractable dysmenorrhea or pelvic pain are found to have endometriosis (Cramer and Missmer, 2002; Donnez *et al*, 2002a).

The most common risk factors for endometriosis are the presence of dysmenorrhea (which may be interpreted as a symptom of disease), early menarche (defined as $\leq$ age 11) and a shorter cycle length (defined as $\leq$ 27 days). Some studies suggest that a longer-lasting and heavier menstrual flow may favor the development of endometriosis, and outflow obstruction of menstruation might alone be sufficient to

cause endometriosis (Cramer and Missmer, 2002; Donnez *et al*, 2002a).

Other risk factors include taller height, alcohol and caffeine consumption, family history, and dioxin-like polychlorinated biphenyls (PCB) or dioxin exposure. Higher parity and weight, regular exercise and smoking may decrease the risk for developing endometriosis (Cramer and Missmer, 2002; Donnez *et al*, 2002a).

1.3. Clinical symptoms and diagnosis

Endometriosis is the cause of dysmenorrhea (pelvic pain during menstruation), dyspareunia (pain during sexual intercourse), chronic pelvic pain and infertility in more than 35% of women of reproductive age. Endometriotic lesions on the anterior cul-de-sac or bladder flap may give rise to dysuria, urinary urgency, pollakiuria, suprapubic pain and hematuria when there is invasion through the bladder wall. Involvement of the ureter may occur with or without obstruction, and may lead to the loss of a kidney.

The most common form of extraperitoneal endometriosis involves the rectovaginal septum, and may present as intestinal obstruction, rectal pain, distension, diarrhea, constipation and/or rectal bleeding. Lung and chest wall endometriosis usually present as pneumothorax, hemothorax or hemoptysis.

Endometriosis is most often diagnosed in women of reproductive age. Although rare, endometriosis may occur during the menopause. In

asymptomatic women, the diagnosis of endometriosis ranges from 2 to 22% of reproductive-age women (Donnez *et al*, 2002a; Murphy, 2002).

Time to diagnosis can be very long (mean 11.7 years in the USA and 8 years in the UK) because of the variability in symptoms and signs, and confusion with other disorders. The gold standard for diagnosis of pelvic disease is surgical assessment by laparoscopy or laparotomy and biopsy. A scoring system has been developed to assess the extent of disease: the revised American Society for Reproductive Medicine (rASRM) classification of endometriosis (Anonymous, 1997).

Decreased quality of life may result not only from the symptoms of pelvic pain and infertility, but also from the side effects of various medical and surgical treatments. For women with pain, surgery commonly provides temporary relief, but symptoms recur in up to 75% of women within 2 years, and further surgery is needed in many cases (Giudice and Kao, 2004).

1.4. Peritoneal, ovarian, and rectovaginal endometriosis are three distinct entities

The most widely accepted hypothesis on the origin of endometriosis is Sampson's theory of retrograde menstruation (Sampson, 1927). According to this theory, endometriosis originates from endometrial cells regurgitated through the Fallopian tubes (Figure 3).
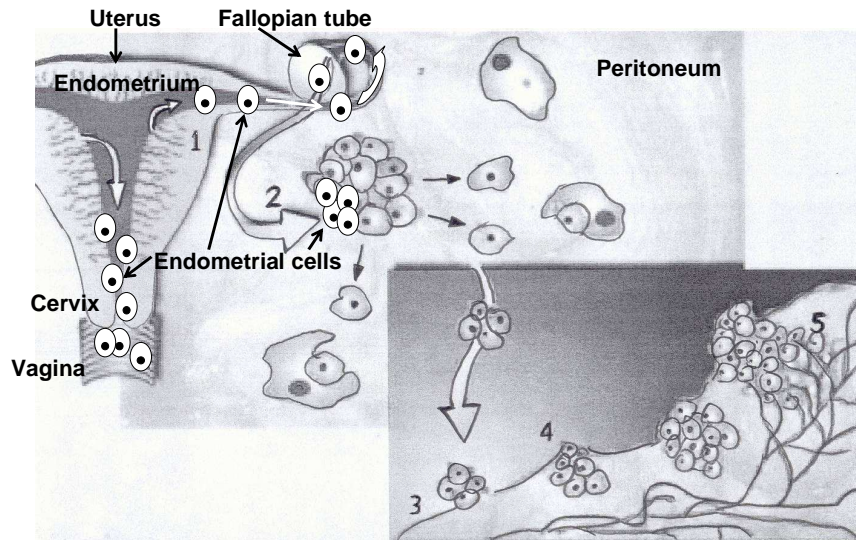
Figure 3: Sampson's theory. 1) During menstruation, the superficial layer of the endometrium is sloughed off through the uterine cervix and vagina, but retrograde flow of the menstrual slough through the Fallopian tubes may occur. 2) Regurgitated endometrial cells arrive in the peritoneal cavity. 3) Endometrial cells adhere to the peritoneum. 4) The peritoneum is invaded by endometrial cells. 5) Establishment of a blood supply, cell proliferation and resistance to apoptosis in endometrial cells all play a role in the development of peritoneal endometriotic lesions (adapted from Groothuis *et al*, 2005).

In 1996, Donnez *et al* distinguished two different types of endometriosis. The rectovaginal endometriotic nodule was then classed as a distinct entity from peritoneal endometriosis. One year later, Nisolle and Donnez (1997) published a definitive guide for future publications on endometriosis: "Peritoneal endometriosis, ovarian endometriosis, and adenomyotic nodules of the rectovaginal septum are three different entities". In this paper, peritoneal

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endometriosis was explained by the retrograde menstruation theory, and peritoneal endometriotic lesions were characterized depending on their appearance into red, black or white endometriotic lesions (Figure 4).
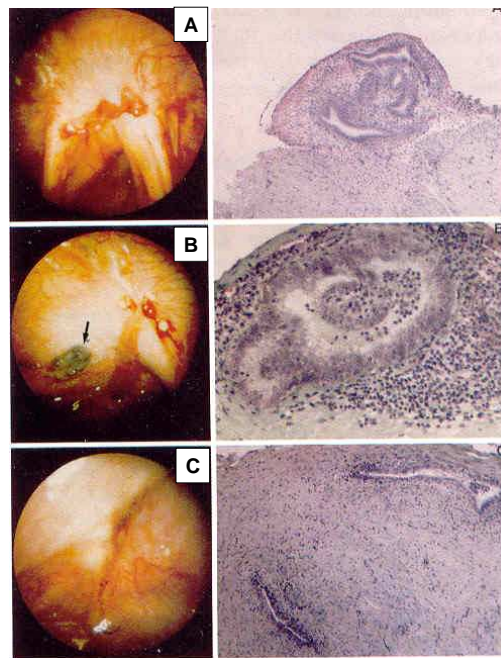
Figure 4: Peritoneal endometriosis. (A) Red endometriotic lesion at laparoscopy. Numerous glands with active epithelium and abundant stroma are found on the peritoneal surface. (B) Typical black endometriotic lesion: combination of glands, stroma and intraluminal debris. (C) White endometriotic lesion: occasional retroperitoneal glandular structures and scanty stroma (Nisolle and Donnez, 1997).

Morphologic and morphometric data showed similarities between eutopic endometrium and red peritoneal lesions, and the evolution theory, demonstrating the transition from red to black and then to white lesions, was proposed (Figure 5).

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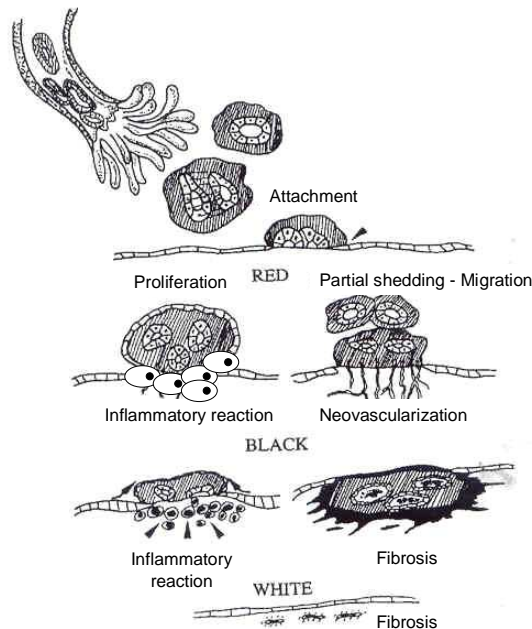
Figure 5: Evolution theory of peritoneal endometriosis (adapted from Nisolle and Donnez, 1997).

Concerning the pathogenesis of ovarian endometriosis, the presence of mesothelial invagination in continuum with endometriotic tissue suggested that metaplastic histogenesis of ovarian endometriotic lesions occurs, but endometriotic cysts may also originate from invagination of superficial endometriotic implants. Regarding the origin of rectovaginal nodules, immunocytochemical results showed poor differentiation and hormonal independence of these lesions and indicated a close relationship with their mesodermal Müllerian origin. Histologically, scanty endometrial-type stroma and glandular epithelium were found to be disseminated in muscular tissue, showing

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striking similarities to adenomyomas or adenomyotic nodules, originating in the retroperitoneal space (Donnez and Squifflet, 2004). Others suggested that rectovaginal nodules could originate from deep-infiltrating endometriosis from the pelvic peritoneal cavity (Koninckx and Martin, 1992). Hematological spread of endometrial cells and metaplasia of different cell types were also proposed as theories to explain extrapelvic endometriosis in the lungs or other organs.

The importance of discriminating between these three different entities was duly confirmed during subsequent years of research into endometriosis, since most studies on endometriosis following this publication focused their objectives distinguishing between peritoneal endometriosis, ovarian endometriosis and adenomyotic nodules of the rectovaginal septum.

For the purposes of this study, research was conducted into peritoneal endometriosis.

1.5. Origin and establishment of peritoneal endometriotic lesions

Sampson's theory (Figure 3) explains the origin of endometriosis as the consequence of reflux of endometrial fragments through the Fallopian tubes during menstruation, with subsequent implantation and growth on the peritoneum (Sampson, 1927). This is the most widely accepted theory to explain the occurrence of peritoneal endometriosis.

Sampson's transplantation theory is supported by the high prevalence of pelvic endometriosis in girls with congenital obstruction of their menstrual outflow. Menstruation is often longer and heavier in women with endometriosis (Sanfilippo *et al*, 1986; Darrow *et al*, 1993; Vercellini *et al*, 1997; Vinatier *et al*, 2001) and cycles tend to be shorter (Arumugam and Lim, 1997). Furthermore, obstruction of antegrade menstruation and the formation of uteropelvic fistulas are effective ways to induce endometriosis in primate models, clearly indicating that increased retrograde menstrual flow increases the risk of developing endometriosis (Te Linde and Scott, 1950; D'Hooghe *et al*, 1994).

Retrograde menstruation may occur in up to 90% of patients with patent tubes (Halme *et al*, 1984), but not all of them will develop endometriosis. Peritoneal reflux appears to be necessary, but not sufficient in itself, for the development of peritoneal endometriosis. In the majority of women, the peritoneal environment is able to eliminate refluxed endometrial tissue, but this cleansing mechanism seems to be deficient in women who develop endometriosis. Thus, the origin and establishment of endometriosis is explained by the ability of endometrial cells to survive, adhere, invade tissues, create a blood supply and proliferate outside their eutopic location (Van Langendonckt *et al*, 2002a; Giudice and Kao, 2004).

1.5.1.   Adhesion to the peritoneal surface

Menstrual endometrium with viable endometrial cells may therefore enter the pelvic cavity and come into close contact with the peritoneum, creating constant apposition of endometrium to mesothelium. *In vitro,* the mesothelial lining can be damaged by the contents of the menstruum, and the extracellular matrix (ECM) is thus exposed, forming adhesion sites for endometrial tissue (Dunselman *et al*, 2001). *In vivo,* endometrial cells have been shown to adhere to intact mesothelium in a murine model (Nisolle *et al*, 2000a, 2000b; Donnez and Nisolle, 2001). Adhesion of endometrial cells to the peritoneum observed *in vivo* could be explained by damage or remodeling of the mesothelium induced by the menstrual tissue, as demonstrated in *in vitro* experiments (Koks *et al*, 2000; Demir Weusten *et al*, 2000).

Integrins and cadherins are two important mediators of cell-cell and cell-matrix adhesion and these cell adhesion molecules are found on the endometrium, in menstrual effluent and in endometriotic tissue samples (Van der Linden *et al*, 1994, 1995; Bridges *et al*, 1994; Kim and Yamada, 1997; Regidor *et al*, 1998).

1.5.2.   Invasion of the epithelium

The initial establishment of endometriosis is an invasive event requiring ECM breakdown. Endometrial stromal and epithelial cells have been implicated in the attachment and early invasion of the peritoneum. Two families of proteolytic enzymes are involved in this

process: MMPs and the plasminogen/plasmin activation system (Rodgers *et al*, 1994). Once endometrial tissue adheres to the ECM, endometrial metalloproteinases begin active remodeling of the ECM, leading to endometrial invasion of the submesothelial space of the peritoneum (Nisolle *et al*, 2000a; Dunselman *et al*, 2001; Witz *et al*, 2003). MMPs have been shown to play a key role in the initiation of menstruation and contribute to the implantation and further invasion of seeded endometriotic explants (Marbaix *et al*, 1996; Singer *et al*, 1997; Henriet *et al*, 2002). A direct correlation between MMP-1 expression and the activity of endometriotic foci has been reported (Kokorine *et al*, 1997). Suppressing MMP secretion with progesterone treatment or blocking enzyme activity with the tissue inhibitor of metalloproteinase-1 (TIMP-1) was able to prevent the formation of ectopic lesions in a nude mouse model of endometriosis (Bruner *et al*, 1997).

The second family of proteases involved in peritoneal invasion of endometrial tissue is the plasminogen/plasmin system. Plasminogen and urokinase-type plasminogen activator (uPA) have been detected at higher concentrations in ectopic than eutopic endometrium (Sillem *et al*, 1998). Another molecule that has been shown to enhance stromal cell invasion is hepatocyte growth factor (HGF). HGF stimulates stromal cell invasion partly through uPA induction (Yoshida *et al*, 2004).

1.5.3. Angiogenesis

The development of a blood supply through angiogenesis has been proposed as an essential process for the establishment, survival and growth of endometriotic lesions. Angiogenesis involves proliferation, migration and extension of endothelial cells, adherence of these cells to the ECM, remodeling of the ECM, and formation of a new lumen (Folkman and Shing, 1992; Donnez *et al*, 1998). Menstrual effluent contains high concentrations of vascular endothelial growth factor (VEGF), one of the main factors stimulating angiogenesis. Increased levels of angiogenic factors and angiogenic activity have been detected in the peritoneal fluid of women with endometriosis (Oosterlynk *et al*, 1993; Koninckx *et al*, 1998; McLaren, 2000). The presence of VEGF has been confirmed in epithelial glands, stromal cells and macrophages from ectopic endometrium. The high VEGF levels found in endometriotic lesions could produce an increase in the subperitoneal vascular network and facilitate the initial development and maintenance of endometriotic lesions (McLaren *et al*, 1996; Shifren *et al*, 1996; Donnez *et al*, 1998).

1.5.4. Endometriotic lesion survival

Cell proliferation is a fundamental process in the development of endometriotic lesions. In the nude mouse model of endometriosis, Nisolle *et al* (2000a) demonstrated that shortly after attachment of stromal cells, a rearrangement of epithelial and stromal cells occurs, leading to the development of endometriotic lesions and cystic glands

within just 5 days. Extensive proliferation was observed in glandular cells as early as 3 days after transplantation. The growth of endometriotic tissue can be regulated by ovarian steroid hormones and a number of cytokines and growth factors such as interleukin (IL)-6, IL-8, tumor necrosis factor α (TNF-α), and HGF (Harada *et al*, 2001; Khan *et al*, 2003).

HGF, its receptor c-Met, the proliferating cell nuclear antigen, and survivin mRNA have all been found to be expressed in peritoneal endometriotic lesions (Khan *et al*, 2003; Fujino *et al*, 2006), conferring an antiapoptotic and mitogenic phenotype to these lesions. Ectopic and regurgitated endometrial cells show resistance to apoptosis, contributing to the development of endometriotic lesions (García-Velasco and Arici, 2003; Beliard *et al*, 2004).

1.5.5. Evolution, activity and appearance of peritoneal endometriotic lesions

Peritoneal lesions go through various stages and have a range of aspects, appearing as red, black or white lesions (Figures 4 and 5), red lesions being the most active in terms of cell proliferation, inflammatory response and vascularization (Nisolle and Donnez, 1997; Donnez *et al*, 1998; Khan *et al*, 2004; Van Langendonckt *et al*, 2004). The morphology of red peritoneal lesions is similar to that of eutopic endometrium, suggesting that these lesions are the first stage of early implantation of endometrial glands and stroma (Figure 5). After partial shedding, red lesions can regrow constantly. This

shedding induces an inflammatory reaction, provoking scarification, and the lesions become black. The subsequent fibrosis leads to areas of white opacification that are inactive (Nisolle and Donnez, 1997).

Other studies have shown differential inflammatory reactions, vascularization, and mitotic and apoptotic activity in red and black endometriotic lesions. The more intense inflammatory response observed in red endometriotic implants is substantiated by findings that macrophage infiltration, MMP-1 mRNA expression and macrophage migration inhibitory factor (MIF) are increased in red endometriotic lesions compared to black lesions (Kokorine *et al*, 1997; Kats *et al*, 2002; Khan *et al*, 2004). A recent study by Lawson *et al* (2007) showed higher expression of IL-1 receptor type 1 (IL-1RI) in red endometriotic lesions than in black and in white lesions. IL-1RI could be one of the factors responsible for the increased inflammatory response in red endometriotic lesions.

The observation of a more extensive vascularization pattern in red endometriotic lesions is supported by the fact that VEGF and microvessel density have been found to be higher in red endometriotic lesions than black lesions (Donnez *et al*, 1998; Khan *et al*, 2003).

Concerning the mitotic and apoptotic activities of peritoneal endometriotic lesions, hepatocyte growth factor (HGF), its receptor c-Met, the proliferating cell nuclear antigen, and survivin mRNA expression and concentrations have also been found to be greater in

red endometriotic lesions than black lesions (Khan *et al*, 2003; Fujino *et al*, 2006).

All these findings confer to red endometriotic lesions a major status of inflammation, vascularization, proliferation and resistance to apoptosis.

1.6. Endometriosis: a multifactorial disease

Endometriosis is nowadays considered to be a multifactorial disease. In addition to the etiopathogenic factors previously mentioned, genetic, environmental, hormonal, immunologic and oxidative stress factors have also been implicated in the establishment, development, maintenance and progression of endometriotic lesions (Figure 6).

1.6.1. Genetic factors

Endometriosis is a condition showing hereditary tendencies and a polygenic/multifactorial etiology has been suggested. A number of candidate genes have been proposed with potential biological plausibility (cytochrome P450, N-acetyl transferase 2, glutathione-S-transferase, galactose-1-phosphate uridyl transferase, ER, PR, AR, p53, PTEN, PPARγ2). Some of these genes point to abnormalities in detoxification enzymes, which could lead to vulnerability to environmental stimuli, while others (tumor suppressor genes) are associated with malignant transformation (Simpson and Bischoff, 2002; Giudice and Kao, 2004). New findings reported by Zondervan *et al* (2007) provide significant evidence that a locus with near-

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Mendelian autosomal inheritance on chromosome 7p is linked to endometriosis.
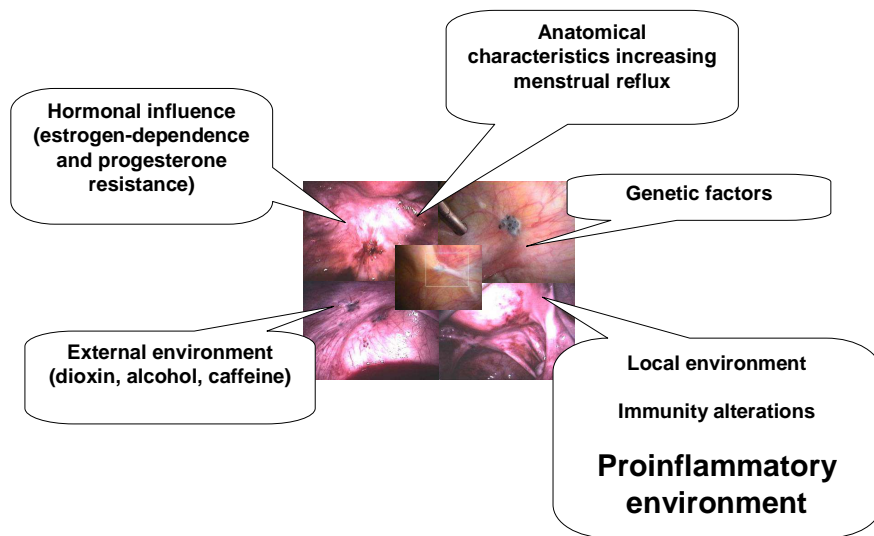
Figure 6: Endometriosis: a multifactorial disease.

1.6.2. Environmental exposure

Endocrine-disrupting compounds, such as dioxins or dioxin-like PCBs, could play a role in the establishment or development of endometriosis. Experimental data in monkeys exposed to dioxin have shown development of endometriosis in a dose-dependent manner. Belgium, with the highest levels of dioxin pollution in the world, has the highest incidence of endometriosis, as well as the highest prevalence of severe endometriosis (Rier *et al*, 1993; Koninckx *et al*,

1994; Heilier *et al*, 2005). Heilier *et al* (2005) provided the first epidemiological evidence of a clear association between increased dioxin and dioxin-like PCB impregnation and the risk of endometriosis.

1.6.3. Hormonal factors

Endometriosis is known to be an estrogen-dependent disease, but progesterone (P) resistance has also been postulated to be a mechanism of progression of endometriosis (Bulun *et al*, 2006).

Endometriotic lesions show high estradiol (E) biosynthesis and low E inactivation compared to endometrium from unaffected women. The central enzyme in the biosynthesis of estradiol is aromatase, which catalyzes the conversion of androgens to estrogens. Aromatase is normally absent from endometrium, but abnormal expression of aromatase has been demonstrated in endometriotic lesions and, at much lower levels, the endometrium of women with endometriosis. In addition, inactivation of E by 17β-hydroxysteroid dehydrogenase 2 is impaired due to a lack of this enzyme in glandular cells in endometriosis, leading to increased local concentrations of E (Noble *et al*, 1997; Zeitoun *et al*, 1998, 1999; Zeitoun and Bulun, 1999)

Expression of both PR-A and PR-B isoforms appears to be dysregulated in the endometrium of endometriosis patients, while in endometriotic implants, only the A isoform is expressed. The presence of PR-A (inhibitory isoform) and absence of PR-B (stimulatory

isoform) has been proposed to explain P resistance in endometriotic lesions. Since P inhibits and E stimulates the growth of endometriotic lesions, partial progesterone resistance and increased local concentrations of E have been suggested as important elements in the development of endometriosis (Lessey *et al*, 1989; Nisolle *et al*, 1997; Berqvist and Ferno, 1999; Attia *et al*, 2000).

1.6.4. Immunologic factors

Local immunity has been found to be altered in peritoneal endometriosis. Abnormal levels of multiple cytokines and chemokines are present in the peritoneal fluid of endometriosis patients, resulting in a proinflammatory local environment. Activated macrophages showing reduced surface expression of scavenger receptors have been reported to be increased in the peritoneal cavity of women with endometriosis, leading to increased cytokine production, but decreased phagocytic activity. Increased cytokine levels and altered macrophage function can promote the survival and growth of ectopic endometrial cells (Harada *et al*, 2001; Sidell *et al*, 2002; Van Langendonckt *et al*, 2002a).

Cytokines, chemokines, adhesion molecules and/or growth-promoting factors involved in endometriosis-associated inflammatory reaction include IL-1, IL-8, TNF-α, IFN-γ (interferon-γ), RANTES (regulated on activation, normal T cell expressed and secreted), MCP-1 (monocyte chemoattractant protein-1), ICAM-1, HGF, MMP-1 and MIF. In the peritoneal fluid of women with endometriosis, these

molecules can originate from endometriotic implants, macrophages or other immune cells (Harada *et al*, 2001; Chegini, 2002; Gazvani and Templeton, 2002; Giudice and Kao, 2004).

Natural killer cell activity is also altered in the peritoneal fluid of women with endometriosis, leading to decreased surveillance of ectopic tissue (Oosterlynck *et al*, 1991).

1.6.5. Oxidative stress

Several studies suggest that oxidative stress is a constituent of the endometriosis-associated inflammatory response. Retrograde menstruation carries erythrocytes, apoptotic endometrial tissue and cell debris into the peritoneal cavity, components which, in conjunction with peritoneal macrophages, contain highly pro-oxidant factors such as heme and iron, well known inducers of oxidative stress. Reactive oxygen species (ROS) may play a role in regulating expression of genes encoding immunoregulators, cytokines and cell adhesion molecules implicated in the pathogenesis of endometriosis (Arumugam and Yip, 1995; Murphy *et al*, 1998).

Previous studies conducted by our team at the Université Catholique de Louvain have shown increased iron levels in peritoneal tissue, macrophages, pelvic fluid and ectopic endometrial tissue from patients with endometriosis (Van Langendonckt *et al*, 2002a, 2002b, 2002c, 2004). Increased levels of iron are associated with tissue damage. Iron can act as a catalyst to potentiate oxygen toxicity by generating a wide

range of free radical species. A recent publication by our group and PhD thesis entitled "Study of the potential involvement of iron in the pathogenesis of peritoneal endometriosis" by S. Defrère (2006) established that iron overload contributes to the further growth of endometriosis by promoting epithelial cell proliferation in endometriotic lesions (Defrère *et al*, 2006).

1.7. Current therapies and novel therapeutic strategies in endometriosis

Current therapies

Endometriosis therapy has essentially three main objectives: to reduce pain, to increase the possibility of pregnancy and to delay recurrence for as long as possible.

A number of drugs have been shown to diminish endometriosis-associated pain: non-steroidal anti-inflammatory drugs (NSAIDs), combined oral contraceptives (COCs), danazol, gestrinone, medroxyprogesterone acetate and gonadotropin-releasing hormone agonists (GnRH-a). They show similar efficacy, but their side effects and cost profiles differ (Donnez *et al*, 2002a, 2002b, 2004).

There is general agreement that visible endometriosis should be removed at the time of surgery. Danazol or GnRH-a therapy for 6 months after surgery reduces endometriosis-associated pain and delays recurrence rates at 12 and 24 months (Donnez *et al*, 2002a; Kennedy *et al*, 2005).

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In moderate and severe endometriosis-associated infertility, the combined approach (operative laparoscopy with GnRH-a for 3 months) is considered as first-line treatment. After surgery, the mean pregnancy rate is about 50% (Donnez *et al*, 2002a, 2002b). *In vitro* fertilization (IVF) is indicated as second-line treatment in patients who fail to become pregnant naturally after surgical treatment or as first-line treatment if tubal function is compromised or male factor infertility is established (Donnez *et al*, 2002a; Kennedy *et al*, 2005).

The rectovaginal adenomyotic nodule, considered as a distinct entity, requires a surgical approach because of the poor response to any medical therapy (Koninckx and Martin, 1992; Donnez *et al*, 2002a, 2002b; Donnez and Squifflet, 2004).

Novel therapeutic strategies

Novel therapeutic strategies are based on many of the molecular targets mentioned earlier, including hormonal therapies, inflammatory and immunological factors, antioxidants, metalloproteinases and angiogenesis. Emerging hormonal therapies include aromatase inhibitors, GnRH antagonists, progestogen agonists, selective PR modulators, selective ER modulators and ER-β agonists. Novel non-hormonal therapies include antiangiogenic agents and anti-inflammatory agents such as selective cyclooxygenase-2 (COX-2) inhibitors, TNF-α inhibitors and PPAR-γ agonists (Giudice and Kao, 2004; D'Hooghe *et al*, 2006; Mihalyi *et al*, 2006).

The absence of any wholly successful medical or surgical therapy, the unacceptable side effects provoked by long-term medical treatments, the epidemiological and social impact of endometriosis and the disability suffered by each individual patient make continued research in this field absolutely indispensable to fully understand the mechanisms implicated in endometriosis development, and to discover new central targets for the treatment of this debilitating disease.

In the next section, the transcription factor nuclear factor-kappaB (NF-κB) will be introduced. This factor plays a key role in the immune and inflammatory response, modulates cell proliferation and apoptosis in many cell types and has also been implicated in angiogenesis. Interest in this molecule in the context of endometriosis has been steadily growing in recent years.

The involvement of NF-κB in peritoneal endometriosis and its potential as a novel central therapeutic target for the treatment of endometriosis will be investigated.

2. The transcription factor nuclear factor-kappaB (NF-κB)

2.1. The NF-κB-signaling system

More than 24000 articles appear in PubMed when the word NF-kappaB is keyed in, giving us some idea of the magnitude and importance of this transcription factor in scientific research, since its discovery by Sen and Baltimore (1986) over 20 years ago.

NF-κB is a transcription factor that plays a crucial role in inflammation, immunity, cell proliferation and apoptosis. It is involved in several pathologies and is known to play a key role in the transduction of proinflammatory signals. The NF-κB pathway has been shown to be modulated in a cell-specific manner and to interact with other transcription pathways, providing a complex variety of cellular responses to its activation (Viatour *et al*, 2005; Perkins, 2007).

NF-κB activation

NF-κB peptides are assembled through the dimerization of five subunits: p50/p105 (NF-κB1), p52/p100 (NF-κB2), p65 (RelA), c-Rel and RelB (Figure 7), the most extensively studied dimer being p50/p65 (Karin, 2006).
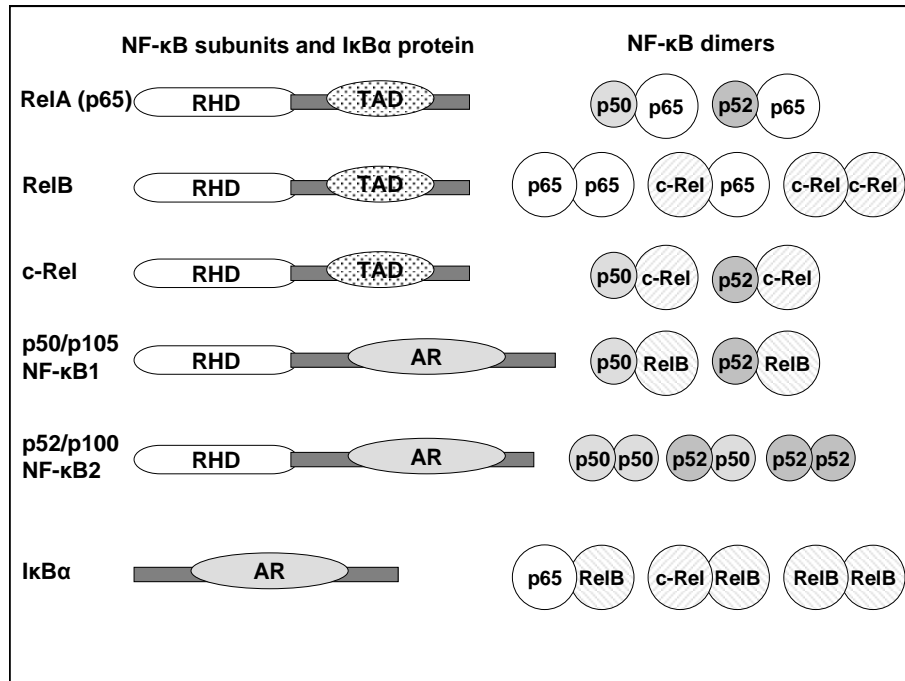
Figure 7: NF-κB subunits and dimers and IκBα protein. RHD: Rel-homology domain, responsible for DNA binding, dimerization and association with IκB proteins. TAD: transcriptional activation domain. AR: ankyrin repeats, responsible for cytoplasmic retention and inhibition of NF-κB. The peptides p50 and p52 derive from the precursor proteins p105 and p100 and lack a TAD; therefore dimers in the 5th row lack TADs. Dimers in the final row are not able to bind to DNA (adapted from Karin *et al*, 2004 and Hoffmann and Baltimore, 2006).

NF-κB dimers are located mostly in the cytoplasm in an inactive form, binding to specific NF-κB inhibitors, IκB proteins, which prevent NF-κB-DNA binding (Figure 8). Diverse stimuli may activate the IκB kinase (IKK) complex, which phosphorylates NF-κB-coupled IκB peptides, inducing their polyubiquitination and rapid degradation by the 26S proteasome. Thus, liberated NF-κB dimers, capable of binding to DNA, translocate to the nucleus, activating the transcription of

several target genes (Baldwin, 1996; Barnes, 1997; DiDonato *et al*, 1997; Lawrence *et al*, 2001; Karin *et al*, 2004; Hoffmann and Baltimore, 2006). NF-κB activity is constitutive in B cells and in some monocyte cell lines but, in most other normal cells, it is very low or undetectable (Rice and Ernst, 1993). Most tumor cell lines and tumor tissues derived from patients with different types of cancer also show constitutive activation of NF-κB (Aggarwal, 2004).

NF-κB activation pathways

Different NF-κB activation pathways have been described (Figure 8). The classic or canonical pathway is induced by proinflammatory stimuli such as pathogen-derived lipopolysaccharide (LPS) and cytokines such as TNF-α and IL-1. This pathway is dependent on IKKβ activation, which phosphorylates IκBα and mainly results in the activation of p50/p65 heterodimers.

The alternative or non-canonical pathway is induced by several stimuli (CD40 and lymphotoxin-β receptors, B-cell-activating factor of the TNF family, LPS and latent membrane protein-1) and is dependent on IKKα activation, resulting in the activation of p52/RelB heterodimers.

The canonical pathway is principally responsible for regulating inflammation and control of proliferation and apoptosis of lymphoid cells during the immune response, being fast-acting (responds within minutes) and reversible, due to the presence of multiple negative

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feedback mechanisms. The non-canonical pathway is related to lymphoid organogenesis and responds slowly (over hours and days), ensuring long-lasting NF-κB activity.

Atypical pathways may be IKK-dependent or -independent, activating casein kinase-II or tyrosine kinase depending on the stimuli, and may induce differentially modified forms of NF-κB subunits with distinct functions (Hoffmann and Baltimore, 2006; Perkins, 2007).
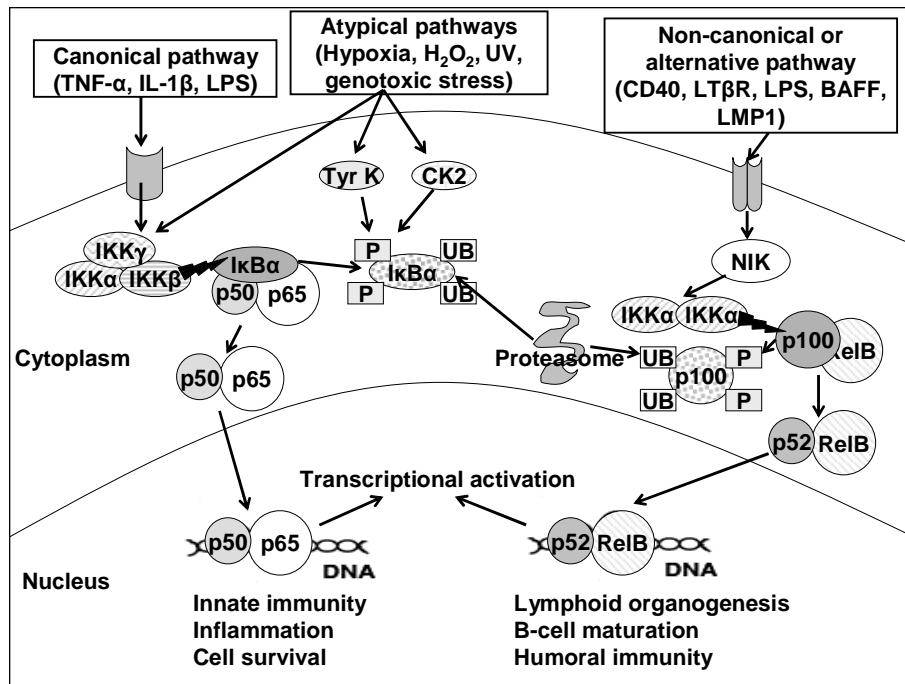
Figure 8: NF-κB activation pathways. UV: ultraviolet radiation, LTβR: lymphotoxin-β receptor, BAFF: B-cell-activating factor of the TNF family, LMP1: latent membrane protein-1, Tyr K: tyrosine kinase, CK2: casein kinase-II, P: phosphorylation, UB: ubiquitination, NIK: NF-κB-inducing kinase (adapted from Bonizzi and Karin, 2004, and Perkins, 2007).

NF-κB-regulated genes

NF-κB transcriptional activity regulates many genes implicated in inflammation (IL-1, IL-6, IL-8, iNOS, COX-2), immune regulation (IFN-γ, TNF-α, RANTES, ICAM-1), apoptosis and cell proliferation (Bax, Fas, Fas-L, cyclin D1, c-Myc, EGF) and angiogenesis (VEGF) (Figure 9).

Figure 9: NF-κB-regulated genes with positive and negative feedback modulating the NF-κB pathway.

NF-κB regulation involves negative feedback inhibition through NF-κB-directed synthesis of IκBα, p100 and/or p105 (Chiao *et al*, 1994; Aggarwal, 2004; Karin, 2006) (Figure 9). IκBα enters the nucleus,

removes NF-κB from the DNA, and then exports it to the cytoplasm, restoring the pool of inactive NF-κB/IκBα complexes (Arenzana-Seisdedos *et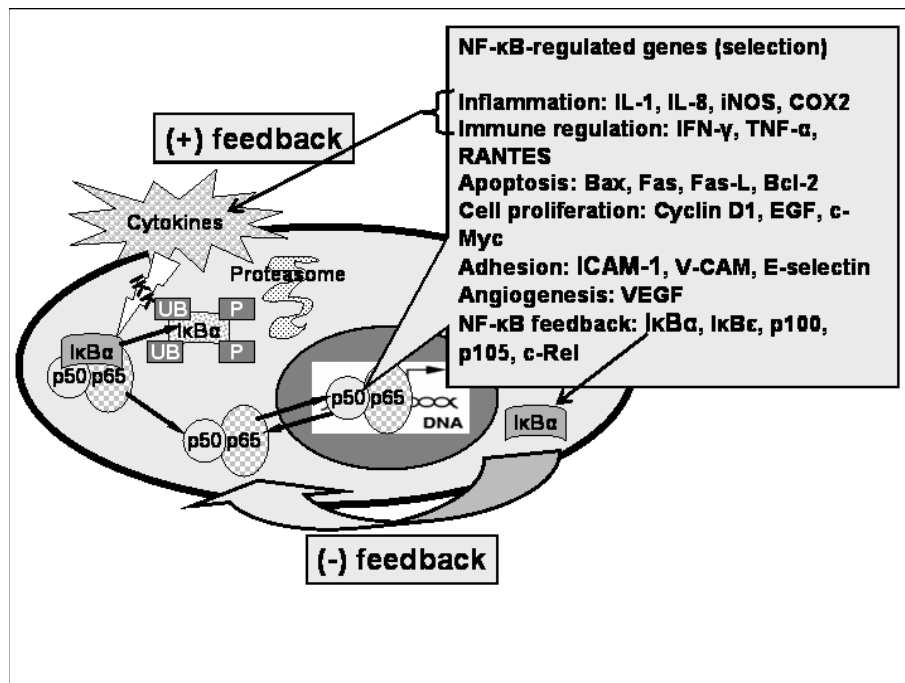 al*, 1997). In pre-B or mature B cells, there is a balanced state between the rates of degradation and synthesis of IκBα proteins (Rice and Ernst, 1993).

2.2. NF-κB, immunity and inflammation

Multiple functions have been ascribed to NF-κB in the immune system, including regulation of the development, differentiation and survival of immune cells, as well as regulation of local and systemic immune activities (Hoffmann and Baltimore, 2006).

NF-κB activation has been implicated in many inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, lupus, inflammatory bowel disease, sepsis, and others (Neurath *et al*, 1998; Abraham, 2003; Roman-Blas and Jimenez, 2006).

A central role of NF-κB in the immune system involves modulation of inflammation. As previously mentioned, NF-κB is activated by proinflammatory stimuli, such as TNF, IL-1, IL-12, MCP-1 and RANTES, inducing expression of multiple genes encoding proinflammatory and/or immunomodulatory cytokines, chemokines, growth factors and adhesion molecules like IL-1, IL-6, IL-8, iNOS, COX-2, IFN-γ, TNF-α, RANTES and ICAM-1. Almost all genes induced by proinflammatory stimuli are upregulated by NF-κB (www.nf-kb.org; Hoffmann and Baltimore, 2006).

Both initiation and resolution of inflammation have been linked to NF-κB transcriptional activity. This could be related to the negative feedback mechanisms regulating the activity of NF-κB. In macrophages, phosphorylation of the RelA subunit of NF-κB by IKKα may lead to the degradation of promoter-bound RelA, which results in control of inflammation. IKKα activation could therefore both induce and limit NF-κB activity. In addition, NF-κB activation involving p50/p50 dimers, which lack transcriptional activation domains, has been observed during resolution of inflammation (Lawrence *et al*, 2001, 2005; Han and Ulevitch, 2005).

2.3. NF-κB, cell proliferation and apoptosis

Suppression of NF-κB in different cancer cell lines and in *in vivo* cancer models inhibits proliferation, causes cell cycle arrest, and leads to apoptosis, demonstrating the crucial role of NF-κB in cell proliferation and survival (Aggarwal, 2004).

A number of cytokines regulated by NF-κB act as growth factors in various types of cancer. IL-1β, TNF, IL-6 and EGF have been reported to act as growth factors in acute myeloid leukemia, Hodgkin's lymphoma, cutaneous T cell lymphoma, gliomas and multiple myeloma, among others. IL-1, TNF and EGF stimulate cell proliferation through activation of NF-κB. Inhibition of NF-κB activation can reduce cyclin D1 activity, a positive regulator of G1-to-

S-phase progression, leading to delayed cell cycle progression (Yamamoto and Gaynor, 2001; Aggarwal, 2004).

Mice embryos lacking RelA die due to massive apoptosis of the liver, secondary to the proapoptotic effects of TNF (Beg *et al*, 1995).

NF-κB directly activates several antiapoptotic genes such as cellular inhibitors of apoptosis (c-IAP1, c-IAP2 and IXAP), TNF receptor-associated factors (TRAF1 and TRAF2) and the Bcl-2 homolog A1/Bfl-1. Cellular inhibitors of apoptosis block activation of caspase-8, an initiator protease, involved at an early stage in stimulating the apoptotic pathway. NF-κB directly induces expression of A1/Bfl-1 by binding to specific sites in its promoter, which prevents cytochrome *c* release from mitochondria and activation of caspase-3 in the B lymphocyte, playing an important role in the survival of this cell type (Grumont *et al*, 1998; Yamamoto and Gaynor, 2001).

In T lymphocytes, NF-κB may stimulate expression of the Fas ligand, activating induced cell death, which suggests that NF-κB proteins have different effects on apoptosis in different cell types (Kasibhatla *et al*, 1999; O'Connor *et al*, 2000).

2.4. NF-κB, invasion and angiogenesis

Expression of proteins involved in cellular invasion and angiogenesis can be upregulated by NF-κB transcriptional activation.

MMPs, uPA and tissue plasminogen activator (tPA) proteases, responsible for cellular invasion, have been found to be positively

regulated by NF-κB. MMP-1, -2, -3, -9 and -13 expression is modulated by NF-κB in different cell types (Farina *et al*, 1999; Baldwin, 2001; Andreakos *et al*, 2003). The uPA promoter contains an NF-κB binding site that directly induces uPA expression by RelA, and uPA is transcriptionally activated by IL-1 and TNFα through induction of NF-κB activity (Novak *et al*, 1991; Wang *et al*, 1999b; Aggarwal, 2004).

Inflammatory cells such as macrophages and neutrophils produce growth factors and chemokines known to be regulators of angiogenesis, including MCP-1, IL-8, TNF and VEGF, which are upregulated by NF-κB activation (Chilov *et al*, 1997; Kiriakidis *et al*, 2003).

2.5. NF-κB, estrogens and progesterone

ER and PR have been found to engage in antagonistic cross-talk with NF-κB in different cell types, but these interactions appear to be complex and probably specific to cell type (Stein and Yang, 1995; Kalkhoven *et al*, 1996; McKay and Cidlowski, 1998, 1999; Kalaitzidis and Gilmore, 2005). In osteoblasts, NF-kB interacts with ER, resulting in mutual repression. This interaction reduces NF-kB binding to its promoter (as shown for IL-6 gene regulation) as well as ER binding to promoters with ER binding sites This interaction thus leads to reduced transcriptional activity of NF-κB- and ER-dependent promoters (Stein and Yang, 1995). Several mechanisms can explain the interaction

between ER and NF-κB: 1) repression of NF-κB-DNA binding by physical association with ER; 2) regulation of IκB expression by estrogen; 3) repression of NF-κB signaling by ER-dependent increased expression of PR (McKay and Cidlowski, 1999).

A study by Kalkhoven *et al* (1996) showed mutual repression between hormone-activated PR (A and B isoforms) and RelA in HeLa cells (epithelial cells), COS-1 cells (kidney fibroblast cells) and T47D cells (human breast tumor cell lines) *in vitro*. This mutual repression could have resulted from direct physical interaction between the two proteins, forming inactive complexes capable of binding to DNA, but unable to interact with essential co-factors or the basal transcriptional machinery.

McKay and Cidlowski (1998), working on COS-1 cell cultures, evaluated the effect of increasing amounts of RelA on PR-B-, AR- and ER-mediated transactivation. They proved that these steroid hormone receptors are inhibited by RelA in a similar way to glucocorticoid receptor (GR), theoretically concluding that a similar direct physical interaction or competition for co-factors underlies transrepression in each case. Contrary to the previously cited findings of Stein and Yang (1995) and Kalkhoven *et al* (1996), PR-B and ER had no effect on RelA activation in this study, failing to show any mutual antagonism between these molecules. The authors explained these discrepancies by the existence of cell type-specific differences in steroid receptor/RelA interactions, which is consistent with the hypothesis

that co-factor competition is involved in NF-κB/steroid receptor antagonism.

2.6. NF-κB and oxidative stress

ROS have also been implicated in the activation of NF-κB (Schreck *et al*, 1991; Flohé *et al*, 1997). Basically, three observations have been made involving cellular oxidative signaling in NF-κB activation: 1) antioxidants such as pyrrolidinedithiocarbamic acid (PDTC) and N-acetylcysteine (NAC) inhibit activation of NF-κB; 2) administration of hydrogen peroxide (H_2O_2) to certain cells stimulates activation of NF-κB; 3) most agents activating NF-κB initiate formation of ROS. In murine macrophages, mitochondrial ROS are required for hypoxic activation of NF-κB and TNF-α gene transcription, but not for LPS-dependent activation of NF-κB (Chandel *et al*, 2000; Hayakawa *et al*, 2003).

Nonetheless, the contribution of redox regulation to NF-κB activation is controversial because of conflicting findings. Activation of NF-κB by H_2O_2 is highly cell type-dependent (Anderson *et al*, 1994; Bowie *et al*, 1997; Li and Karin, 1999; Korn *et al*, 2001). Thus, NF-κB activation does not appear to be a universal response to oxidative stress. Hayakawa *et al* (2003) showed that both NAC and PDTC inhibit NF-κB activation independently of antioxidative function in epithelial and fibroblast cell lines respectively, and that endogenously produced ROS do not lead to NF-κB activation in epithelial cell lines.

ROS are important in mediating cross-talk between c-Jun NH_2-terminal kinase (JNK) and TNF-dependent NF-κB activation. TNF receptor-1 ligation activates NF-κB and JNK signaling, two pathways with opposing biological roles. JNK activation promotes apoptosis via the mitochondrial-dependent pathway, and NF-κB activation promotes cell survival by upregulating expression of antiapoptotic members of the Bcl2 family and caspase inhibitors, and by down-regulating JNK activation. Indeed, some studies have reported that NF-κB down-regulates JNK activation by suppressing TNF-induced ROS accumulation. TNF-dependent NF-κB activation can lead to expression of antioxidant enzymes, which may explain the more efficient ROS clearance (Bubici *et al*, 2004; Papa *et al*, 2004; Sasazuki *et al*, 2004; Gloire *et al*, 2006).

3. The NF-κB pathway in endometriosis *in vitro*

3.1. NF-κB and endometrium

The first study to investigate NF-κB in human endometrium was published in 2000 (Laird *et al*). Human endometrial epithelial and stromal cells showed expression of the RelA subunit of NF-κB, as evaluated by immunohistochemical staining. RelA expression was stable in stromal cells during the menstrual cycle, with a slight increase during the periovulatory phase. In epithelial cells, RelA staining varied greatly throughout the cycle: in the proliferative phase, RelA expression was absent or very weak, but in the secretory phase, staining was more extensive, reaching maximum levels at the time of implantation and decreasing again at the end of the cycle. In this study, addition of the NF-κB nuclear translocation inhibitor SN-50 and the proteasome inhibitor MG132 to endometrial epithelial cell cultures inhibited IL-1α and TNF-α stimulated IL-6 and leukemia inhibitory factor (LIF) production, supporting the hypothesis that NF-κB regulates IL-1α- and TNFα-induced expression of LIF and IL-6 in endometrial epithelial cells.

King *et al* (2001) detected RNA expression of IκBα, IKKα, IKKβ, IKKγ and NF-κB-inducing kinase (NIK) in human endometrium at different phases of the cycle. IKKα and NIK were immunolocalized mainly in the glandular epithelium and endothelium of the

endometrium. IκBα mRNA levels were found to be significantly greater in perimenstrual endometrium. In this study, IκBα was positively regulated by P in culture of endometrial epithelial cells. The authors postulated that P withdrawal at the end of the menstrual cycle activates NF-κB, which could be responsible for the increase in IκBα expression during the perimenstrual period. Both the p50 subunit of NF-κB and IκBβ have been found to be expressed in endometrial epithelial and stromal cells, as shown by immunohistochemical analyses (Page *et al*, 2002).

Slight activation of the NF-κB pathway has been demonstrated in non-stimulated endometrial epithelial and stromal cell cultures (Tamura *et al*, 2002a; Han and Sidell, 2003).

Expression of the proinflammatory mediators RANTES, COX-2 and MIF was shown to be upregulated by NF-κB activation in IL-1β- or TNF-α-stimulated endometrial stromal cells *in vitro* (Tamura *et al*, 2002b; Cao *et al*, 2005, 2006). The non-specific NF-κB inhibitor, sulindac, was tested in endometrial stromal cell cultures, showing a reduction in TNF-α-induced RANTES expression as a result of decreased NF-κB activation (Wieser *et al*, 2005). The positive modulation of RANTES, COX-2 and MIF supports NF-κB as a proinflammatory transcription factor in endometrial stromal cells. Moreover, NF-κB plays a role in cell growth, angiogenesis and tissue remodelling, sustained by the upregulation of MIF in these cells.

Concerning oxidative stress in endometrial stromal cell cultures, NF-κB has been found to induce expression of manganese superoxide dismutase (Mn-SOD) in response to TNF-α. Mn-SOD protects cells by scavenging superoxide radicals. The involvement of NF-κB in Mn-SOD induction by TNF-α may be considered a self-defense mechanism against TNF-α-mediated oxidative stress (Sugino *et al*, 2002). E and P withdrawal in endometrial stromal cells *in vitro* stimulates COX-2 and prostaglandin F2α expression through ROS-induced NF-κB activation, suggesting a possible mechanism for menstruation (Sugino *et al*, 2004).

A controversial study on endometrial epithelial cell lines showed that the antiprogestin mifepristone (RU486) inhibits endometrial epithelial cell growth and induces apoptosis through stimulation of NF-κB (Han and Sidell, 2003).

3.2. Induced NF-κB activity in endometriotic stromal cell cultures

NF-κB-dependent transcriptional activation of proinflammatory genes, such as IL-1 or TNF-α, may provide positive feedback to the pathway, self-perpetuating the inflammatory response and providing possible mechanisms to resist apoptosis and stimulate cell proliferation in endometriotic lesions (Guo, 2007). This hypothesis is based on knowledge of the NF-κB pathway and the inflammatory response in normal endometrium, ectopic endometrial stromal cells in culture and other type of cells or pathologies. A few *in vitro* studies have shown

that NF-κB can be activated in endometriotic stromal cells by known inducers of the canonical NF-κB pathway, such as IL-1β, TNF-α or LPS (Lebovic *et al*, 2001; Sakamoto *et al*, 2003; Iba *et al*, 2004). The chemoattractant chemokine RANTES was up-regulated by IL-1β via NF-κB activation in endometriotic stromal cells, possibly producing a feed-forward regulatory loop and increasing macrophage recruitment (Lebovic *et al*, 2001). IL-8 and IL-6 expression was induced by TNF-α, probably through NF-κB activation in endometriotic stromal cells (Sakamoto *et al*, 2003; Yamauchi *et al*, 2004). LPS-induced IL-8 production and endometriotic stromal cell proliferation were reduced by an NF-κB inhibitor *in vitro* (Iba *et al*, 2004). Sulindac, thalidomide, GnRH-a, P and progestational compounds have all been tested in endometriotic stromal cell cultures, showing a reduction in RANTES and TNF-α-induced IL-8 expression as a result of decreased NF-κB activation (Sakamoto *et al*, 2003; Horie *et al*, 2005; Wieser *et al*, 2005; Yagyu *et al*, 2005). Non-stimulated endometriotic stromal cells *in vitro* have shown slight NF-κB activation (Horie *et al*, 2005; Wieser *et al*, 2005).

To date, there is no direct evidence of constitutive NF-κB-DNA binding activity in endometriosis *in vivo*. Furthermore, the involvement of the NF-κB pathway in the development of endometriotic lesions and the inflammatory response in endometriosis *in vivo*, and the effect of NF-κB activation or inactivation on cell

proliferation and apoptosis in endometriosis, have not been previously studied.