MOLECULAR PATHWAYS REGULATED BY THE ETV5 TRANSCRIPTION FACTOR IN THE INVASION OF ENDOMETRIAL CANCER

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"Breathe" (Pink Floyd)

Breathe, breathe in the air
Don't be afraid to care
Leave but don't leave me
Look around and chose your own ground
For long you live and high you fly
And smiles you'll give and tears you'll cry
And all you touch and all you see
Is all your life will ever be

Run, run rabbit run
Dig that hole, forget the sun,
And when at last the work is done
Don't sit down, it's time to dig another one
For long you live and high you fly
But only if you ride the tide
And balanced on the biggest wave
You race toward an early grave.

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ABREVIATIONS

AD – Amino-terminus Domain

AJCC – American Joint Committee on Cancer

BM – Basement Membrane

BMI – Body Mass Index

BRCA – Breast Cancer gene

ChIP – Chromatin ImmunoPrecipitation

Ct – Carboxy-terminus

COM1 – Candidate Of Metastases 1

CTNNB1 – β-catenin

D – D-score

EBS – ETS Binding Sites

EC – Endometrial Carcinoma

EEC – Endometrioid Endometrial Carcinoma

EGD – Endometrial Glandular Dysplasia

EGF – Epidermal Growth Factor

EH – Endometrial Hyperplasia

EIC – Endometrial Intraepithelial Carcinoma

EIN – Endometrial Intraepithelial Neoplasia

EMT – Epithelial Mesenchymal Transition

ER – Estrogen Receptors

ER – Endoplasmic Reticulum

FIGO – Federation Internationale de Gynecologie d'Obstetrique (International Federation of Gynecology and Obstetrics)

FC – Fold Change

FSH – Follicle Stimulating Hormone

G – Grade

GFP – Green Fluorescent Protein

GnRH – Gonadotropin Releasing Hormone

GOG – Gynaecologic Oncology Group

H – Hec1A

H&E – Hematoxilin and Eosin

HAT – Histone Acetyltransferase Activity

HG – Hec1A-GFP

HGE – Hec1A-GFP-ETV5

HGEshC – Hec1A-GFP-ETV5 short hairpin Control

HGEshNID1 – Hec1A-GFP-ETV5 short hairpin NID1

HGEshNUPR1 – Hec1A-GFP-ETV5 short hairpin NUPR1

HMG – High Mobility Group

IVIS – *In Vivo* Imaging System

LC-MS/MS – Liquid Chromatography- Mass Spectrometry

LH – Luteinizing Hormone

LOH – Loss Of Heterozygosity

LPS – Lipopolisaccharide

MMP – Matrix Metallopetidase (or Metalloproteinase)

MSI – Microsatellite Instability

NEEC – Non-Endometrioid Endometrial Carcinoma

NID1 – Nidogen 1

NLS – Nuclear-Targeting Signal

NUPR1 – Nuclear Protein 1

P – p-value

PCR – Polymerase Chain Reaction

PR – Progesterone Receptors

RIN – RNA Integrity Number

RT – RetroTranscription

RTqPCR – Real Time quantitative Polymerase Chain Reaction

SEER – Surveillance, Epidemiology and End Results

SEGO – Sociedad Española de Ginecología y Obstetrícia (Obstetrics and Gynecology Spanish Society)

SUMO – Sumoylation

TEM7 – Tumour Endothelial Marker 7

TMA – Tissue MicroArray

TVU – TransVaginal Ultrasonography

UICC – Unio International Contre Cancrum (International Union Against Cancer)

UPSC – Uterine Papillary Serous Carcinoma

USNC – United States National Cancer

WB – Western Blot

WHO – World Health Organization

INTRODUCTION

1 The endometrium

1.1 Anatomy of the uterus

The uterus or womb is the female genital organ responsible for providing the adequate environment for the development of the foetus. It is located in the pelvic cavity between the urinary bladder and the rectum.

The functional uterus is essential to reproduction. During the fertile period, the main role of the uterus is to receive the blastocyst that develops from a fertilized oocyte and to provide a site for implantation. In conjunction with the embryo, the uterus participates in the genesis and development of the placenta, a supportive structure that connects the foetal and maternal circulations for the exchange of nutrients, respiratory gases, waste and various regulatory substances. Prenatal development continues within the uterus until the gestation is completed, at which time the uterus plays an active role in the delivery of the baby.

At the end of the reproductive lifetime of an adult woman, the uterus loses its function and becomes atrophic.

The uterus is a hollow, thick-walled, muscular organ with the shape of an inverted pear, which measures on average 7 cm long, 5 cm wide and 2,5 cm in diameter. It is divided in four anatomic parts (Figure 1):

- The **fundus**, the highest, dome-shaped portion, at the level of entrance of the Fallopian tubes.
- The **body** or corpus, which lies below the fundus and is separated from the cervix by a slight constriction, the isthmus.
- The **isthmus**.
- And the **cervix,** cylindrical, slightly expanded in its middle and about 2 or 3 cm in length. Its canal is spindle-shaped and opens into the vagina [1].

The uterine wall confines the uterus and consists of three strata. From the outer to the inner layer of the uterine cavity we find an outer serosa, the **epimetrium**, followed by a firm, thick, intermediate coat of smooth muscle, the **myometrium**, and finally the **endometrium** (Figure 1).

The endometrium is the glandular inner lining of the uterus, which consits of a membrane lined by a simple cuboidal epithelium.

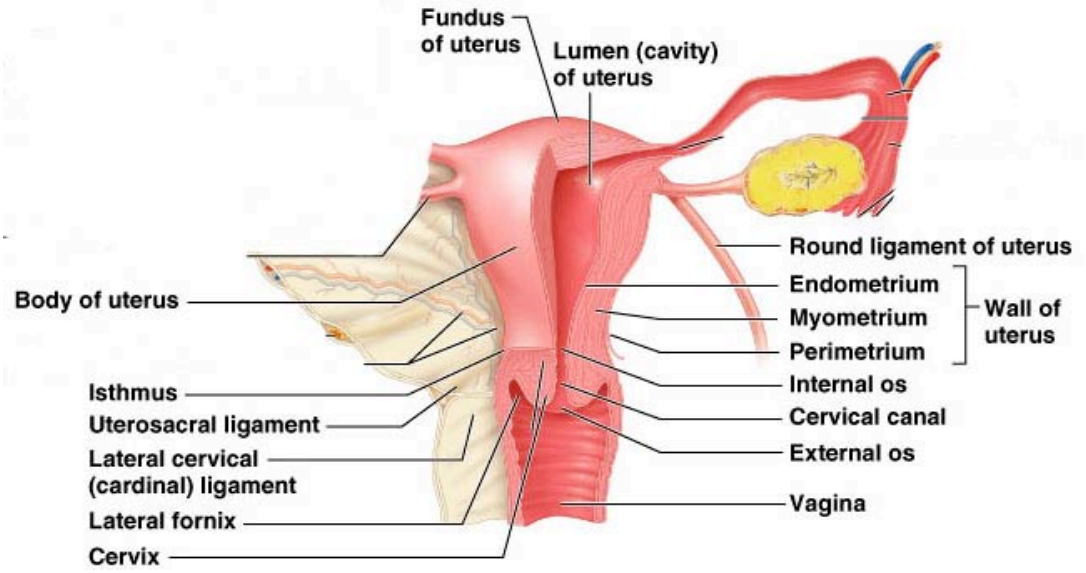


Figure 1. Representative diagram of the uterus.

1.2 Histology of the endometrium and the endometrial cycle

The endometrium consists of a simple columnar epithelium of ciliated and secretory cells and an underlying thick connective tissue stroma. The epithelium is invaginated to form many simple tubular uterine glands that extend through the entire thickness of the stroma. The stromal cells of the endometrium are embedded in a network of reticular fibres. The endometrium is subject to cyclic changes that result in the menstruation [2].

The endometrium can be divided into two layers based on their involvement in the changes of the menstrual cycle: the *basalis* and the *functionalis*.

- The *basalis* is not sloughed off during menstruation, but functions as a regenerative zone for the *functionalis* after its rejection.
- The *functionalis* is the luminal part of the endometrium. It is the site of cyclic changes and it is sloughed off during every menstruation. The cyclic changes are divided in four phases: proliferative (or follicular), secretory (or luteal), menstrual and conception [2].

The significance of cyclic changes in the histology of the endometrium was first recognized by Hitchmann and Adler in 1908. Their observations provided initial evidence to support the concept of sequential pituitary stimulation and ovarian response that prepare the endometrium each month for the implantation and nutrition of a fertilized egg [1].

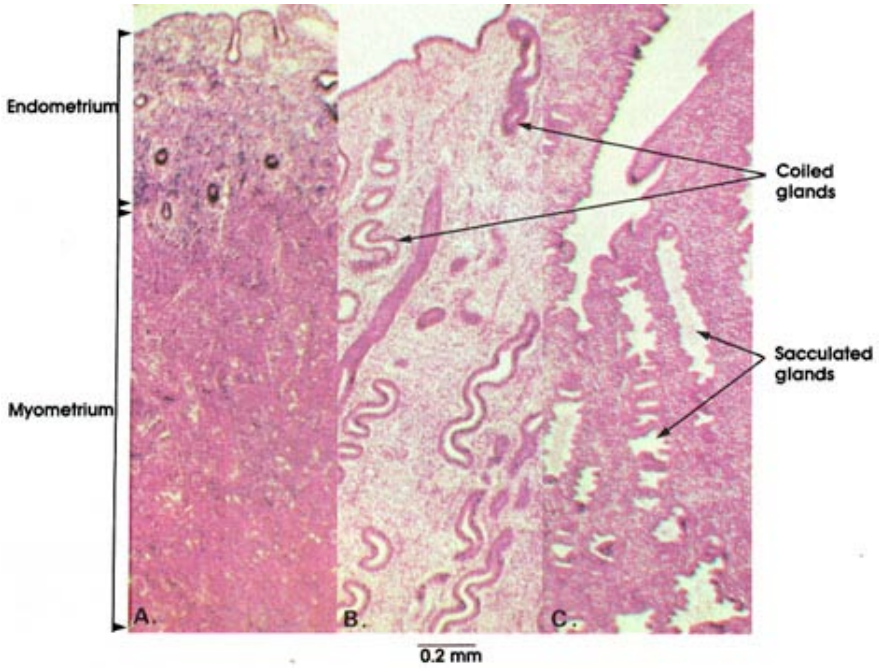


Figure 2. A. Early postmenstrual. B. Proliferative phase. C. Secretory phase. Human, 10% formalin, H. & E., 50x [3].

The changes in the histology of the endometrium during the menstrual cycle, can be described in four phases [1].

I. Proliferative phase

a) Early proliferative phase: directly after cessation of menstruation, it is characterized by a thin, relatively homogenous endometrium. The glands are simple and straight, widely scattered in a dense stroma. The histology of this phase is similar to that of the prepuberal and the postmenopausal endometrium. However, the endometrium of the early proliferative phase is even thinner.

b) Late proliferative phase: much thicker than the early proliferative phase as a result of marked growth of the glands and stroma. The stroma cells of the superficial layer may be separated by oedema. The epithelium is higher and more columnar.

II. Secretory phase

a) Early secretory phase: within 2 or 3 days after the late proliferative stage and induced by progesterone. The endometrium shrinks slightly as the superficial oedema is lost. The total thickness of the uterine mucosa reaches a maximum. The glands adopt a distinctive jagged appearance.

b) Late secretory phase: regressive changes are found coinciding with the decrease and eventual cessation of the function of the *corpus luteum* of the ovaries. The endometrial intracellular oedema is mostly reabsorbed, resulting in a shrinkage in the total thickness of the endometrium. The glandular epithelium appears inactive, the cells are low columnar or cuboidal.

III. Menstruation. It begins with a pooling of blood cells in the intercellular space beneath the surface epithelium. Breaks in the surface occur, and pieces of stroma and broken glands are lifted off. The glands remain relatively inactive, small and simple, as seen in the early proliferative phase.

IV. Conception. The secretory activity of the endometrium is maintained and increased by the vigorous function of the *corpus luteum* of pregnancy. None of the involutional changes described as late secretory are found.

When women's reproductive life ends, the endometrium becomes atrophic. The atrophic endometrium presents at the onset of the menopause. During this transitional phase, the endometrium undergoes important histological modifications and loses its ability to

proliferate and secrete. These changes are mainly consequence of the lack of progesterone stimulation as ovulation ceases to occur. Estrogen stimulation, however, may continue, since the androgens secreted by the menopausal ovaries and adrenal cortices can be converted into estrogens. High estrogen levels, especially of estradiol, are often associated with endometrial hyperplasia, because the hormone binds to the receptor sites in the nuclei of endometrial cells [4, 5].

1.3 Hormonal regulation

The menstrual cycle occurs approximately every 28 days as menstruation follows ovulation, and it is regulated by the hormonal activity of the ovaries. During ovulation, the wall of a follicle ruptures releasing a secondary oocyte that passes into the uterine tube. Ovulation typically occurs from alternate ovaries. If fertilization occurs, mitotic divisions are initiated and the blastocyst implants on the uterine wall. If the egg is not fertilized, the menstrual cycle is initiated, usually 14 days after ovulation. The menstrual cycle is divided into the three phases described before: menstrual, proliferative and secretory (Figure 3) [6].

Reproduction is an event that requires the coordination of peripheral organs with the nervous system to ensure that the internal and external environments are optimal for the successful procreation of the species. This is accomplished through the hypothalamic-pituitary-gonadal axis, which coordinates reproductive behaviour with ovulation.

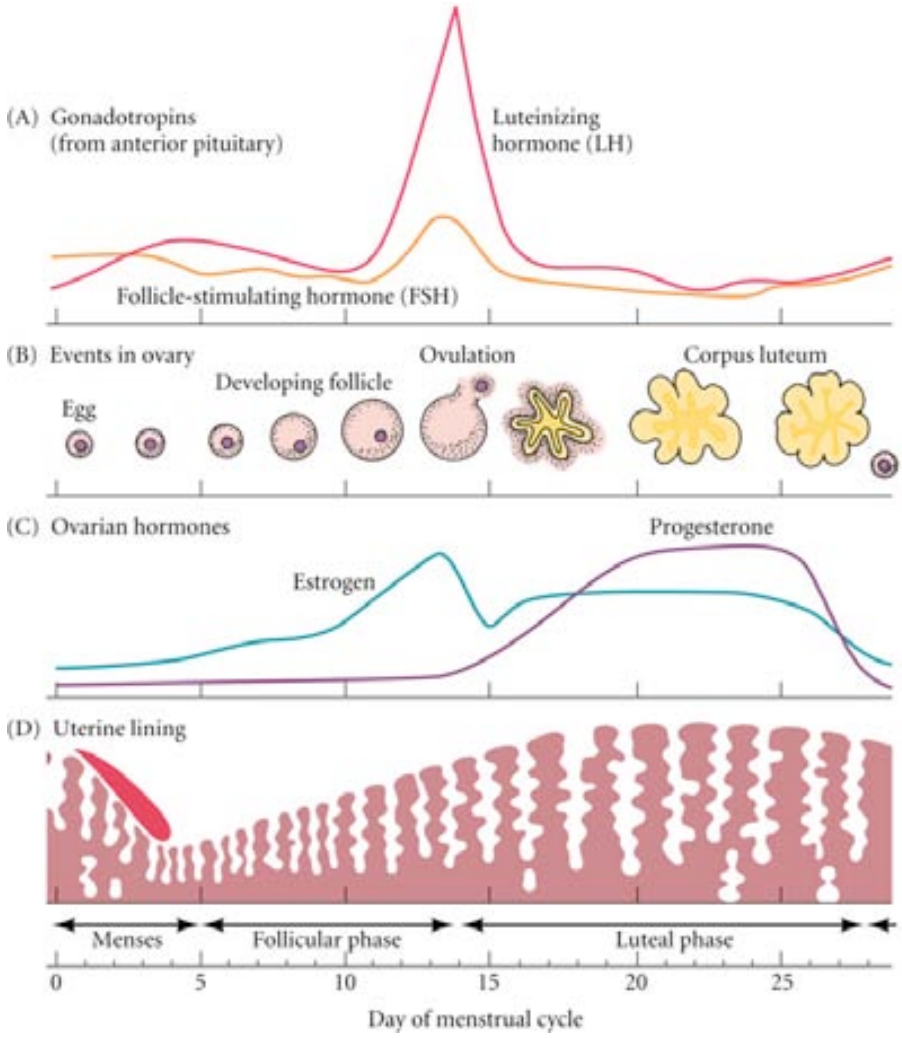


Figure 3. The human menstrual cycle. The coordination of (B) ovarian and (D) uterine cycles is controlled by (A) the pituitary and (C) the ovarian hormones. During the follicular phase, the egg matures within the follicle, and the uterine lining is prepared to receive a blastocyst. The mature egg is released around day 14. If a blastocyst does not implant in the uterus, the uterine wall begins to break down, leading to menstruation [7].

The predominant hormones involved in the menstrual cycle are the gonadotropin releasing hormone (GnRH), the follicle stimulating hormone (FSH), the luteinizing hormone (LH), estrogen and progesterone. GnRH is secreted by the hypothalamus, the gonadotropins FSH and LH are secreted by the anterior pituitary gland, and estrogen and progestin are secreted in the ovary (Figure 4).

In the first part of the cycle (proliferative or follicular phase), the pituitary gland starts secreting increasingly large amounts of FSH. Any maturing follicles in the ovary that have reached a certain stage of development respond to this hormone with further growth and cellular proliferation. FSH also induces the formation of LH receptors on the granulosa cells. Shortly after this period of initial follicle growth, the pituitary gland begins secreting LH. In response to LH, the nuclear membranes of competent oocytes break down, and the chromosomes assemble to undergo the first meiotic division. It is at this stage that the egg will be ovulated.

The two gonadotropins acting together cause the follicle cells to produce increasing amounts of estrogen. As a result of estrogen, the following five major regulator activities in the progression of the menstrual cycle take place:

1. The uterine endometrium starts to proliferate and becomes densely vascularised.
2. The cervical mucus thins out to facilitate sperm entry into the inner portions of the reproductive tract.
3. The number of FSH receptors on the granulosa cells of the mature follicles increases and the pituitary lowers its FSH production. Estrogen also stimulates the granulosa cells to secrete the peptide hormone inhibin, which also suppresses pituitary FSH secretion.
4. At low concentrations, estrogen inhibits LH production, while at high concentrations, LH production is stimulated.
5. At very high concentrations and over long periods, estrogen affects the hypothalamus, causing the secretion of gonadotropin-releasing hormone.

When estrogen levels increase as a result of follicular production, FSH levels decline. LH levels, however, continue to rise while more estrogen is secreted. As estrogen levels increase (days 7 to 10), the granulosa cells continue to grow.

Starting on day 10, estrogen secretion rises sharply. This rise is followed at mid-cycle by an enormous surge of LH and a smaller burst of FSH. Within 10 to 12 hours after the gonadotropin peak, the egg is ovulated.

Following ovulation, the luteal phase of the menstrual cycle begins. Under the continuous influence of LH, the remaining cells of the ruptured follicle become the *corpus luteum*. The *corpus luteum* secretes estrogen, but its predominant secretion is progesterone. This steroid hormone circulates to the uterus, where it completes the job prepares the uterus for blastocyst implantation by stimulating the growth of the uterine wall and its blood vessels. Progesterone also inhibits the production of FSH, thereby preventing the maturation of any more follicles and eggs. If the ovum is not fertilized, the *corpus luteum* degenerates, progesterone secretion ceases, and the uterine wall is sloughed off. With the decline in serum progesterone levels, the pituitary secretes FSH again, and the cycle is repeated. However, if fertilization occurs, the trophoblast secretes luteotropin, a hormone that keeps the *corpus luteum* active and thus high levels of serum progesterone.

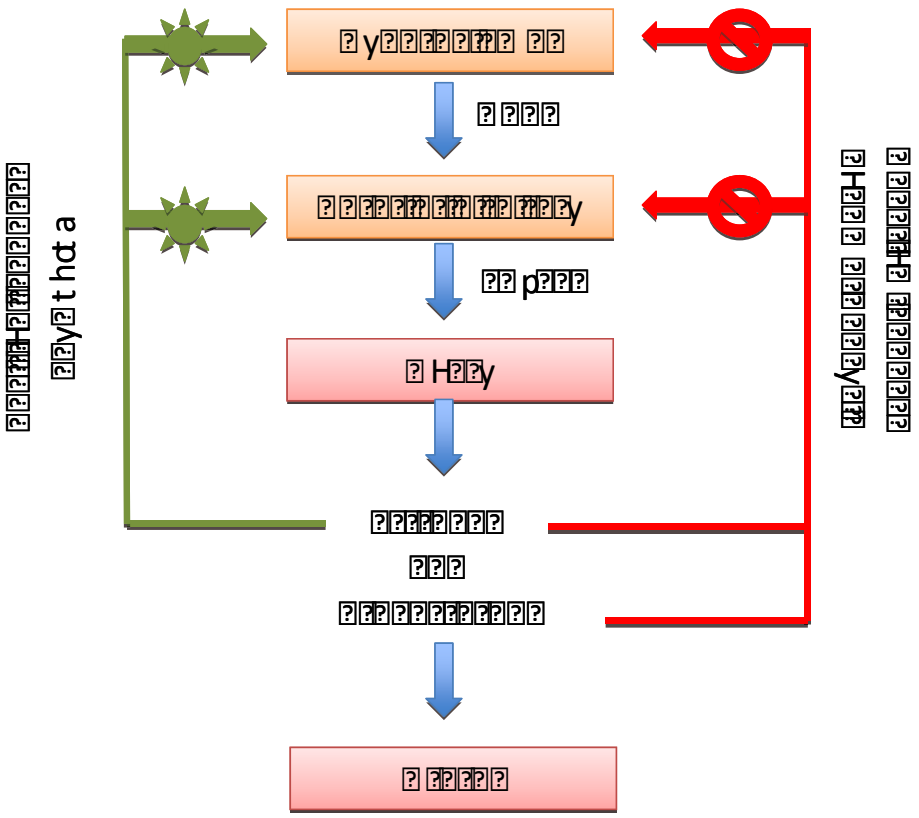


Figure 4. Hypothalamic-pituitary-adrenal axis. Hormonal regulation of the menstrual cycle.

2 Endometrial carcinoma

2.1 Epidemiology

Endometrial carcinoma is the most common malignancy of the female genital tract in developed countries, with approximately 47,130 new cases and 8,010 deaths in 2012 in the United States [8] and 82,500 new cases and 21,700 deaths in 2008 in Europe [9].

Based upon data from the US national cancer database Surveillance, Epidemiology and End Results, the incidence of uterine cancer from 2005 to 2009 was 24.1 per 100,000 women. Incidence rates were higher in whites than in black, Hispanic, or Asian/Pacific Islander women (Figure 5). However, mortality was almost twice as high in black than in white women (Figure 5), possibly due to a higher incidence of aggressive cancer subtypes, as well difficulties regarding access to and quality of healthcare services [10].

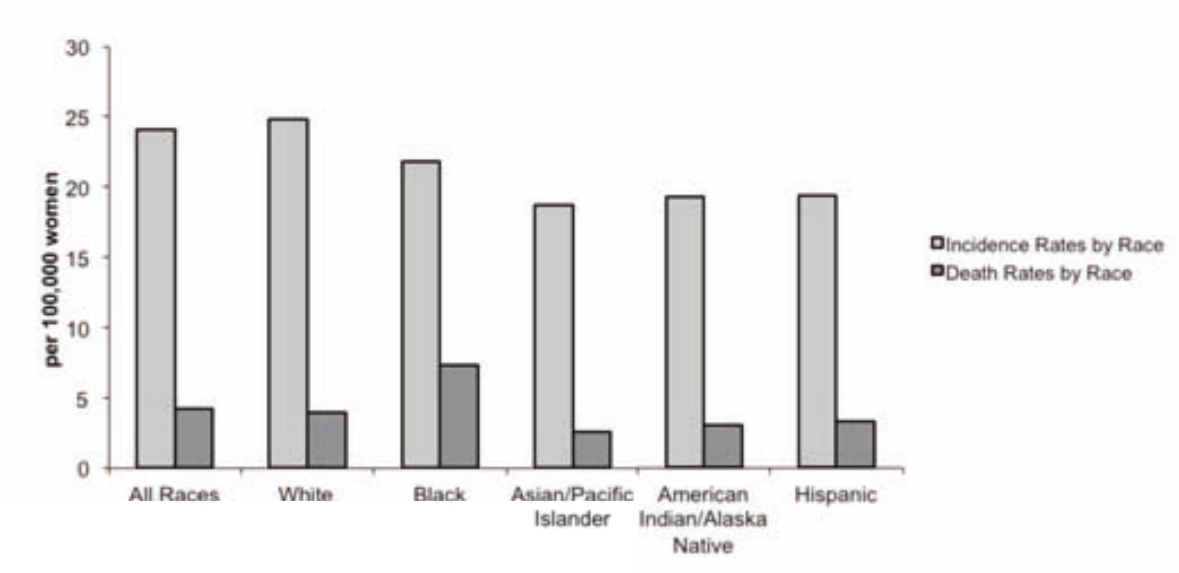


Figure 5. Incidence and Death Rates from the SEER database. Table adapted from the Surveillance, Epidemiology and End Results database.

Endometrial carcinoma usually affects postmenopausal women over 70 of age. The probability of developing endometrial cancer is 1.24% in postmenopausal women [8]. Most affected women are diagnosed in the early stages of the tumour when it is confined to the uterus. Disease-related signs and symptoms, such as abnormal vaginal bleeding, trouble urinating and abdominal pain, prompt women to seek medical advice. The 5-year

survival rate for women with a localized tumour is 96%. This percentage decreases dramatically for women with regional or distant disease (Figure 6).

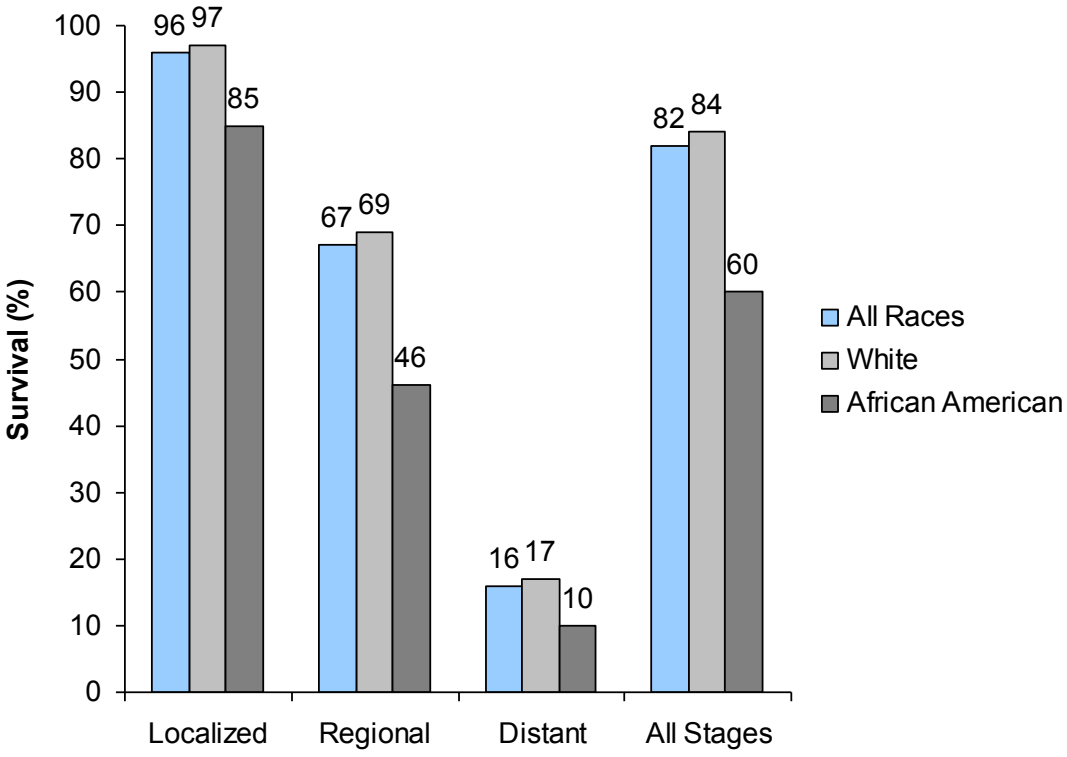


Figure 6. Five-year relative survival rates by race and stage at diagnosis, United States, 2001-2008. Graphic modified from Jemal et al., Cancer Statistics, 2012.

2.2 Risk factors of endometrial carcinoma

Approximately 5% of all EC (endometrial carcinoma) are caused by inherited susceptibility. Although large epidemiologic studies have investigated risk factors for type I endometrioid EC [11-15], little is known about type II (non-endometrioid) EC [16] (see Section 3.1 Dualistic model for EC).

The most important and well-recognized risk factors for type I EC are:

1) Long-lasting unopposed hyperestrogenism

Estrogen exposure promotes cell proliferation and inhibits apoptosis through a complex downstream cascade of transcriptional changes that may include the modulation of tumour suppressor functions. Estrogen may also increase the rate of mutagenesis through free radical formation [17].

a) Exogenous estrogen or estrogen agonist

i) Unopposed estrogen therapy

Multiple studies have shown an increased incidence of endometrial carcinoma, with the relative risk ranging from 1.1 to 15, [18-20] as well as an increase in the incidence of endometrial hyperplasia from 20 to 50%, in women after one year of receiving systemic estrogen therapy without a progestin [21-23]. The risk is related to estrogen dose and duration of use.

ii) Tamoxifen

Tamoxifen is the usual hormonal anti-estrogen therapy for hormone receptor-positive breast cancer in pre-menopausal women, and a standard treatment in breast cancer in post-menopausal women [24]. Tamoxifen is an antagonist of the estrogen receptor in breast tissue. However, in other tissues such as the endometrium, it behaves as an agonist, and can thus be characterized as a mixed agonist/antagonist.

The endometrial activity of tamoxifen appears to depend upon menopausal status [25]. For postmenopausal women, the increased risk of endometrial cancer with tamoxifen use is well-established. This effect is dose and duration-dependent. For premenopausal women, however, there is no evidence that tamoxifen increases the risk of endometrial cancer.

b) Endogenous estrogen

Common reasons for excessive endogenous estrogen are **chronic anovulation** or excessive endogenous conversion of adrenal precursors to estrone and estradiol by adipose cells in **obese women** [26]. Several studies have shown that a

postmenopausal woman's risk of developing endometrial carcinoma is correlated with higher circulating estrogen and androgen levels and lower levels of sex hormone binding globulin, leading to increased steroid hormone activity, compared with unaffected controls [27]. **Estrogen-secreting tumours** are rare, but may also result in endometrial carcinoma.

2) Age

Endometrial carcinoma usually occurs in postmenopausal [8]. After the menopause, the ovaries stop making estrogen and progesterone, but a small amount of estrogen is still made naturally in fat tissue. Estrogen has a bigger impact after the menopause, since the compensatory levels of progesterone produced by the ovaries before the menopause have disappeared and the hyperestrogerism stands unopposed [28].

3) Family history

a) Lynch syndrome

Lynch syndrome (hereditary nonpolyposis colorectal carcinoma syndrome) accounts for most hereditary cases [29].

For women with Lynch syndrome, the lifetime risk of endometrial carcinoma is 27 to 71 % compared with 2.6 % in the general population. Most Lynch syndrome-associated endometrial carcinomas are of endometrioid histology and present at an early stage, similarly to the sporadic endometrial carcinoma.

b) BRCA

Carriers of mutations in the Breast Cancer Gene (*BRCA*) genes are at a high risk of breast and ovarian cancer. Some data suggest that BRCA1 mutations are associated with endometrial carcinoma [30]. However, data from a prospective series suggested that the risk of endometrial carcinoma was significantly elevated only for BRCA mutation carriers treated with tamoxifen [31].

4) Nulliparity and infertility

The risk of endometrial carcinoma is inversely related to parity. Nulliparity and infertility do not appear to be independent risk factors for endometrial carcinoma; the risk factor is probably the high frequency of anovulatory cycles [32].

5) Diabetes and hypertension

Women with diabetes and hypertension have an increased risk of endometrial carcinoma [33]. Comorbid factors, particulary obesity, account for much of this risk [34, 35]. The risk of developing endometrial carcinoma is higher in type 2 than in type 1 diabetes.

6) Breast cancer

A history of breast cancer is a risk factor for the development of endometrial carcinoma. This association has been shown in women treated with tamoxifen. However, the fact that both diseases share some common risk factors, i.e., obesity and nulliparity, also accounts for part of this association.

Some data suggest that women with breast cancer who develop endometrial cancer have an increased risk of having serous endometrial tumours [36, 37].

Regarding type II EC, the **mean age** of affected patients is usually higher han for type I EC [38]. Recently, investigators have shown that a high **intake of folates**, particularly from vitamin supplements, is associated with an increased risk on non-endometrioid endometrial cancer [16].

Another risk factor is the **BMI** (Body Mass Index), as shown in the Cancer Prevention Study II Nutrition Cohort [39]. The study discriminated between type I and type II EC and found BMI to be positively associated with both, although the association was stronger in type I EC.

Further research is needed to clarify the possible etiologic factors for this type of malignancy [40].

2.3 Clinical presentations and diagnosis

Despite the absence of reliable screening tools, endometrial carcinoma is usually diagnosed at an early stage because of the early occurrence of abnormal uterine bleeding during its natural history. Abnormal uterine bleeding is present in approximately 90% of endometrial carcinoma cases. When it occurs in postmenopausal women, it should always be treated as a sign that warrants an evaluation to rule out malignancy [41]. The probability of endometrial cancer in women presenting with postmenopausal bleeding is 5–10%, but

the risk increases with age and risk factors [42]. Other symptoms of EC such as abdominal pain and distension occur later, in the advanced stages of disease [40].

When clinical suspicion of EC exists, an endometrial biopsy must be performed to diagnose or exclude malignancy. Endometrial cancer is usually diagnosed histologically from endometrial tissue obtained with miniature endometrial biopsy devices [43]. When the prevalence of endometrial carcinoma is over 15%, endometrial biopsy is considered the most cost-effective initial strategy. On the other hand, the most cost-effective method for populations in which the prevalence of endometrial carcinoma is lower would be an initial exam with a TVU (transvaginal ultrasonography) followed by endometrial biopsy if any abnormality is detected [44]. TVU normality is defined as a thin symmetrical endometrial line of less than 4–5 mm double endometrial thickness [45, 46]. When assessed by an ultrasound expert, a thin and regular endometrial line is associated with a very low risk of endometrial cancer as long as the endometrium is clearly visualised throughout the uterus; however, a normal hysteroscopic image does not always exclude malignancy [47, 48].

Even though endometrial cancer is usually detected in its initial phases, 20% of the patients present with myometrial invasion and/or lymph node affectation, both indicators of advanced disease related to poor prognosis.

Our group has been interested in the development of molecular markers for screening and detection of early endometrial cancer. The final diagnosis of endometrial cancer is usually done by pathological examination of an endometrial aspirate (20-30%) and by a hysteroscopic-guided biopsy (70-80%). The rate of diagnosis success with hysteroscopy is over 90%, with false positives in the case of precursor lesions of endometrial adenocarcinoma (hyperplasias with atypia) and endometrial polyps with a degree of malignancy. Therefore, amongst the main limitations of current methods of diagnosis we find patient discomfort and the subjective interpretation of visual images.

Our research group has recently identified and validated new molecular biomarkers for the detection of EC in uterine aspirates, as a fluid representative of the primary tumour [49]. To this end, gene expression screening of 52 carcinomatous and 10 normal tissues was performed to identify potential biomarkers, which were subsequently validated in an independent series of 19 tissue samples by RTqPCR (Real Time quantitative Polymerase Chain Reaction) and on 50 carcinoma and non-carcinoma uterine aspirates. We found that

the differential expression of these biomarkers in primary endometrial tumours is correlated to their expression in corresponding uterine fluid samples. We confirmed the utility of these biomarkers on uterine aspirates to differentiate between carcinoma and control samples with high sensitivity and specificity [49].
Our final aim is to develop a minimally invasive and highly sensitive and specific method for the identification of EC that will avoid patient discomfort, since the current methods of diagnosis are based on more invasive techniques. We aim to provide a molecular, precise tool to help gynaecologists reduce the number of unnecessary histeroscopies [49].

2.4 Endometrial preneoplastic lesions

2.4.1 Endometrial hyperplasia

Endometrial hyperplasia is the normal response of the endometrium to estrogenic stimulation. It was thought that a continuum from benign to atypical hyperplasia and ultimately to endometrial carcinoma existed, but this view has now been challenged.

Endometrial stimulation is no longer considered to cause atypia and cancer by itself [40]. Endometrial hyperplasia encompasses a group of abnormalities that include pre-malignant lesions of the endometrium. It is classified according to cellular and structural appearances, and is recognized as an oestrogen-dependent condition. Predisposing factors include therapies resulting in exogenous oestrogenic stimulation, and conditions that cause excessive oestrogen production [50].

The diagnosis is most commonly made in women aged 50 to 54 years and is rarely found in women under 30 years of age [51]. The risk factors for endometrial hyperplasia are the same as those for endometrial carcinoma, since both are oestrogen-dependent conditions [50]. The most common are obesity, diabetes and hypertension in post-menopausal women (classic triad) [52]. In addition, women with Lynch syndrome (hereditary nonpolyposis colorectal cancer) have an increased risk of endometrial hyperplasia [53, 54].

Endometrial hyperplasia is usually asymptomatic or presents as post-menopausal uterine bleeding. Occasionally, women with no abnormal uterine bleeding present with abnormal findings on cervical cytology.

Endometrial hyperplasia is characterized by a thick, tan endometrium measuring up 1 cm [52] and by a proliferation of endometrial glands that present a greater gland-to-stroma ratio than that observed in a normal endometrium [55]. The proliferating glands vary in size and shape and cells may have cytological atypia. Endometrial hyperplasia may be non-neoplastic, as in most simple and some complex hyperplasias, or neoplastic, as in some complex and all complex atypical hyperplasias (see Endometrial Hyperplasia Classification). Neoplastic hyperplasia is a non-obligate precursor of endometrioid EC, the most common form of endometrial carcinoma. Regrettably, the terminology used for endometrial hyperplasia does not reflect its malignant potential [51].

The classical definition and classification of hyperplasia with four categories proposed by the WHO (World Health Organization) has been modified to include the three categories revised by Zanio [56] and most recently, the newest two categories classification (Table 1) [40]. However, the 4-level WHO classification is still the most widely used [57].

Table 1. Different classifications of hyperplasia. Table modified from *Textbook of gynaecological oncology*, 2012 [40].

WHO 1994	Simple Hyperplasia	Complex Hyperplasia	Simple Atypical Hyperplasia	Complex Atypical Hyperplasia
Revised classification (by Zaino)[56]	Simple Hyperplasia	Complex Hyperplasia	Atypical Hyperplasia	
New classification (ISGP)	Hyperplasia		Endometrial Intraepithelial Neoplasia (EIN)	

The WHO classification generally correlates with the risk of progression to endometrial carcinoma. However, a major limitation of this system is the inter-observer variability across pathologists reviewing the same slides [58, 59]. Indeed, the finding of nuclear atypia, the most important indicator of malignant potential, has the lowest level of inter-observer agreement. The International Society of Gynaecological Pathologists' (ISGP)

classification is similar but it introduces the term *endometrial intraepithelial neoplasia* (EIN), which decreases inter-observer variability.

The EIN is based on an epithelial crowding that displaces stroma until stromal volume is less than half of total tissue volume (stroma + epithelium + gland lumen). Stromal volume can be measured using computerized morphometric analysis and assigned a D-score [60, 61]. Using this method, specimens are classified as benign (D>1), indeterminate (0<D<1), or EIN (D<0).

The ISGP system do not correspond directly to specific categories in the WHO system, but some correlation exists. Most simple and some complex hyperplasias fall into the EH (Endometrial Hyperplasia) category. Many complex hyperplasias without atypia and most complex hyperplasias with atypia fall into the EIN category [51].

The ISGP classification system has shown high inter-observer reproducibility and studies have confirmed that EIN correlates with progression to endometrial carcinoma [60]. An acknowledged drawback of the ISGP system is that it groups findings that merit different treatments (hormonal treatment or surgery), ostensibly because of the inability to distinguish levels of severity within the EIN category [61].

The 4-category WHO classification of endometrial hyperplasia is based upon two features:

- The glandular/stromal architectural pattern of the endometrium, described as either simple or complex.
- The presence or absence of nuclear atypia. Nuclear atypia is the presence of nuclear enlargement; the chromatin may be either evenly dispersed or clumped [57]. Atypical endometrial hyperplasia is usually complex, although simple atypical hyperplasia can ve observed sporadically.

These results classify endometrial hyperplasia in four categories:

- Simple hyperplasia without atypia (Figure 7A)
- Complex hyperplasia without atypia (Figure 7B)
- Simple atypical hyperplasia
- Complex atypical hyperplasia (Figure 7C)

Simple atypical hyperplasia is rare, and many reports use the term atypical hyperplasia to refer to all women with either simple or complex atypical hyperplasia.

Women with simple hyperplasia without atypia are least likely to develop endometrial carcinoma, whereas women with complex hyperplasia with atypia are the most likely to develop carcinoma. The presence of nuclear atypia is the finding most frequently associated with carcinoma [51].

A) **B)** **C)**

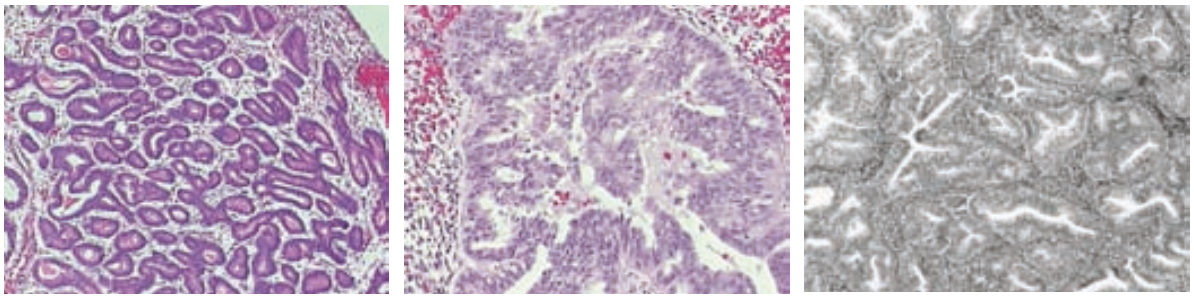


Figure 7. A) Simple hyperplasia stained with H&E (Hematoxilin & Eosin). The glands exhibit extensive crowding. Virtually all glands are tubular and the nuclei are basal. **B) Complex hyperplasia stained with H&E.** The single gland illustrated has a very complex architecture; there are multiple lumens, which interconnect. The cells are tall columnar and some are piled up on top of each other and, in general, do retain their orientation to the lumen [52]. **C) Complex atypical hyperplasia.** The irregularly shaped glands in this case are very closely packed but are still separated by residual endometrial stroma [62].

A drawback of the WHO classification is that the category of complex atypical hyperplasia includes neoplasms bordering on invasive carcinoma and those that are clearly not invasive. Complex atypical hyperplasia is distinguished from grade 1 endometrial carcinoma by the presence of residual endometrial stroma that separates all glands [63].

2.5 Histological classification of endometrial carcinoma.

The current classification of endometrial adenocarcinomas by the International Society of Gynaecological Pathologists and the World Health Organization [64] divides neoplasms according to histologically defined features that describe the appearance of individual neoplastic cells. While pathologists admit that the cell types by themselves do not imply

any particular biological behaviour, it is thought that these features represent acceptable surrogate markers for molecular changes that have yet to be defined [65].

The current histological classification of endometrial carcinomas by WHO [64] are endometrioid adenocarcinoma, mucinous adenocarcinoma, serous adenocarcinoma, clear cell adenocarcinoma, mixed cell adenocarcinoma, squamous cell carcinoma, transitional cell carcinoma, small cell carcinoma and undifferentiated carcinoma.

Table 2. Incidence of endometrial carcinoma types. Adapted from M. Monge [66].

Histological type EC by WHO	Percentage Cases (%)
Endometrioid Adenocarcinoma	80-85
Variant with squamous differentiation	20-25
Villoglandular variant	10
Secretory variant	1-2
Ciliated Cell Variant	8
Mucinous Adenocarcinoma	1-9
Serous Adenocarcinoma	3-10
Clear Cell Adenocarcinoma	1-5
Squamous Cell Carcinoma	0,25-0,5
Undifferentiated Carcinoma	1,5

The most common histological type is the endometrioid adenocarcinoma, which accounts for 80-85% of total endometrial cancers (Table 2).

2.5.1 Endometrioid adenocarcinoma

The endometrioid adenocarcinoma is a primary endometrial adenocarcinoma which contains glands that resemble those of the normal endometrium (Figure 8a).

The endometrioid adenocarcinoma represents a spectrum of histological differentiation from atypical complex hyperplasia to minimally differentiated tumours that can be confused not only with undifferentiated carcinomas but also with various sarcomas. A highly characteristic feature of endometrioid adenocarcinoma is the presence of some

glandular and villoglandular structures lined by simple to pseudostratified columnar cells that have their long axes arranged perpendicularly to the basement membrane with some rather elongated nuclei that are also polarized in the same direction [64].

Endometrial proliferations may exhibit a variety of differentiated epithelial types including squamous/morules, mucinous, ciliated, clear or eosinophilic cells, and architectural variations including papillary formations. These cell types are often called metaplasias and may be encountered in benign, premalignant and malignant epithelia.

Squamous differentiation is a common finding in endometrioid carcinoma [43]. The criteria for squamous differentiation are as follows [64] (Figure 8c):

- Keratinization demonstrated with standard staining techniques
- Intercellular bridges and/or
- Three or more of the following criteria;
    a Sheet-like growth without gland formation or palisading.
    b Sharp cell margins.
    c Eosinophilic and thick or glassy cytoplasm.
    d A decreased nuclear to cytoplasmic ratio as compared with foci elsewhere in the same tumour.

The variants villoglandular (Figure 8b), secretory and ciliated are uncommon and, generally, low-grade tumours. The secretory variant resembles a secretory endometrium, because glycogen vacuoles are present in most of the tumour cells; the ciliated variant has a striking papillary growth pattern [67].

2.5.2 Mucinous adenocarcinoma

The mucinous adenocarcinoma is a primary adenocarcinoma of the endometrium in which most of tumour cells contain prominent intracytoplasmic mucin [64] (Figure 8d).

The mucinous adenocarcinoma may be entirely tubular or cribiform, but it usually presents a papillary architecture. The covering epithelium is made up of multiple layers of columnar cells with intracytoplasmic mucin variably filling the apical portion of the cells, resembling the arrangement of the endocervical epithelium.

The stroma may not be clearly endometrial, and it often contains spindle cells, thin walled blood vessels and variably dense infiltrates of active inflammatory cells. It is a relatively rare form of endometrial adenocarcinoma [68-70].

2.5.3 Serous adenocarcinoma

The serous adenocarcinoma is a primary adenocarcinoma of the endometrium characterized by a complex pattern of papillae with cellular budding. It sometimes contains psammoma bodies (from the Greek *psammos*, sand), a round collection of calcium.
Serous carcinoma (or uterine papillary serous carcinoma (UPSC)) is the most aggressive type of non-endometrioid endometrial carcinoma [71-73]. The histological diagnosis is based on the presence of papillae, covered by highly pleiomorphic tumour cells with frequent mitoses and necrosis (Figure 8e).

2.5.4 Clear cell adenocarcinoma

The clear cell adenocarcinoma is composed mainly of clear cells arranged in solid, tubulocystic or papillary patterns or a combination of those [64].

The clear cell adenocarcinoma of the endometrium is generally recognized by the distinctive, clear cytoplasm of neoplastic cells growing in any combination of solid, glandular, tubulocystic, or papillary configurations (Figure 8f).

The glandular pattern is reminiscent of the tubular glands of the endometrioid adenocarcinoma, whereas the tubulocystic pattern is formed of dilated spherical appearing glands. The papillary pattern is architecturally identical to that of serous carcinoma, with generally short, branching fibrovascular cores, often hyalinized, covered by neoplastic cells [65].

2.5.5 Mixed cell adenocarcinoma

The mixed adenocarcinoma is a tumour composed of an admixture of a type I (endometrioid carcinoma, including its variants; or mucinous carcinoma) and a type II carcinoma (serous or clear cell), in which the minor type must comprise at least 10% of the total volume of the tumour [64].

2.5.6 Squamous cell carcinoma

It is a primary carcinoma of the endometrium composed of the squamous cells of varying degrees of differentiation. Its histological appearance is essentially identical to that of squamous cell carcinoma of the cervix and similarly, it includes a rare verrucous variant [64].

2.5.7 Transitional cell carcinoma

It is a carcinoma in which 90% or more is composed of cells resembling urothelial transitional cells. A lower percentage of transitional cell differentiation would qualify the tumours as a mixed carcinoma with transitional cell differentiation [64].

2.5.8 Small cell carcinoma

It is an endometrial carcinoma that resembles the small cell carcinoma of the lung [64].

2.5.9 Undifferentiated carcinoma

Undifferentiated carcinomas are those lacking any evidence of differentiation [64].

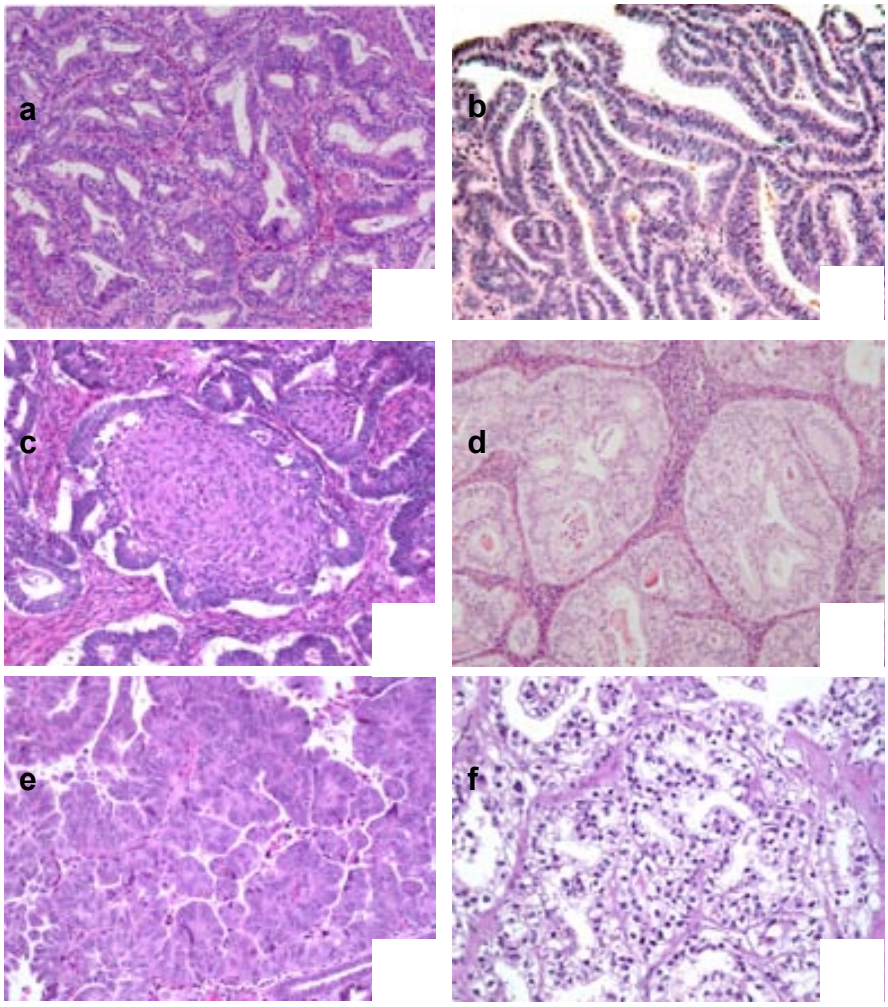


Figure 8. Different histological types of EC. a) Endometrioid adenocarcinoma [74]. b) Villoglandular adenocarcinoma [64]. c) Adenocarcinoma with squamous differentiation. d) Mucinous adenocarcinoma. e) Serous Carcinoma. f) Clear cell adenocarcinoma.

2.6 Staging; FIGO classification

In 1988, the FIGO's (International Federation of Gynaecology and Obstetrics) Committee of Gynaecologic Oncology recommended the surgical staging of endometrial cancer together with the histological verification of the grading and extent of the tumour [75]. The stage of a tumour is a description of the extent to which the cancer has spread. The different stages are numbered progressively from I to IV. The stage generally takes into account the size of a tumour, how deeply it has penetrated within the wall of a hollow organ (intestine, urinary bladder), whether it has invaded adjacent organs, how many regional lymph nodes it has metastasized to (if any), and whether it has spread to distant

organs. Cancer staging is the most important predictor of survival. Moreover, cancer treatment is primarily determined by staging. When tumour grade (G) was identified as an important prognostic feature, many therapists argued for a staging system that would also consider the histological prognostic variables. Histological grade, also called degree of differentiation, refers to how much the tumour cells resemble normal cells of the same tissue, while nuclear grade refers to the size and shape of the nucleus in tumour cells and the percentage of tumour cells that are dividing.

A biopsy or curettage is mandatory to make a final diagnosis. A tumour will be classified as high grade if at least two of the following three criteria are met: more than 50% solid growth; diffusely infiltrative growth, rather than expansive; and tumour-cell necrosis [43]. The tumour grade does not change the staging.

In relation to histological differentiation, endometrial adenocarcinomas are grouped as follows:

• G1: <= 5% of a nonsquamous or nonmorular solid growth pattern.

• G2: 6−50% of a nonsquamous or nonmorular solid growth pattern.

• G3: >50% of a nonsquamous or nonmorular solid growth pattern.

Notes on pathologic grading [75].

- Notable nuclear atypia, inappropriate for the architectural grade, raises the grade of a Grade 1 or Grade 2 tumour by 1.
- In serous and clear cell adenocarcinomas, nuclear grading takes precedent.
- Adenocarcinomas with squamous differentiation are graded according to the nuclear grade of the glandular components.

Endometrial cancer staging was based on physical examination, non-invasive radiographic testing, and measurement of the depth of the uterine cavity. Table 3 shows that clinical staging based on increased uterus size is probably inaccurate and may lead to tumour understaging and possibly to inadequate treatment of a significant proportion of stage I cancers.

Table 3. Definitions of the Clinical Stages in Carcinoma of the Corpus Uteri (1971) (no longer adopted for FIGO classification). Correlation of the International Federation of Gynaecology and Obstetrics (FIGO), Union Internationale Contre le Cancer (UICC), and the American Joint Committee on Cancer (AJCC) nomenclatures [75].

Stage 0	Atypical endometrial hyperplasia. Carcinoma in situ
Stage Ia	The carcinoma is confined to the corpus and the length of the uterine cavity is </= 8 cm
Stage Ib	The carcinoma is confined to the corpus and the length of the uterine cavity is > 8 cm
Stage II	The carcinoma has involved the corpus and the cervix, but has not extended outside the uterus
Stage III	The carcinoma has extended outside the uterus, but not outside the true pelvis
Stage IVa	The carcinoma has extended outside the uterus and involves the mucosa of the bladder or rectum (bullous oedema *per se* does not classify a case as Stage IV)
Stage IVb	The carcinoma has extended outside the true pelvis and spreads to distant organs

In the 1970s and early 1980s several studies in which the surgical-pathological spread pattern was systematically evaluated were carried out, with particular emphasis on the pelvis and paraaortic lymph nodes [76]. In 1971, one of these studies officially incorporated tumour grade into the staging system. The Gynecologic Oncology Group (GOG) inaugurated a pilot study to perform staging laparotomy in the course of initial surgical treatment of patients with clinical stage I endometrial carcinoma. This pilot study highlighted the need for more precise staging, and in 1988 the International Federation of Gynaecology and Obstetrics (FIGO) introduced the requirement for surgical staging of patients with endometrial carcinoma [77].

Subsequently, the FIGO classification was based on surgical stage, histological grading and extent of the tumour (Figure 9A) [75].

A)

Stage Ia *	Tumour limited to the endometrium
Stage Ib *	Invasion of less than half of the myometrium
Stage Ic *	Invasion equal to or more than half of the myometrium
Stage IIa*	Endocervical glandular involvement only
Stage IIb*	Cervical stromal invasion
Stage IIIa *	Tumour invades the serosa of the corpus uteri and/or adnexa and/or positive cytological findings
Stage IIIb *	Vaginal metastases
Stage IIIc *	Metastases to pelvic and/or paraaortic lymph nodes
Stage Iva*	Tumour invasion of bladder and/or bowel mucosa
Stage Ivb *	Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes

*Either G1, G2, G3

B)

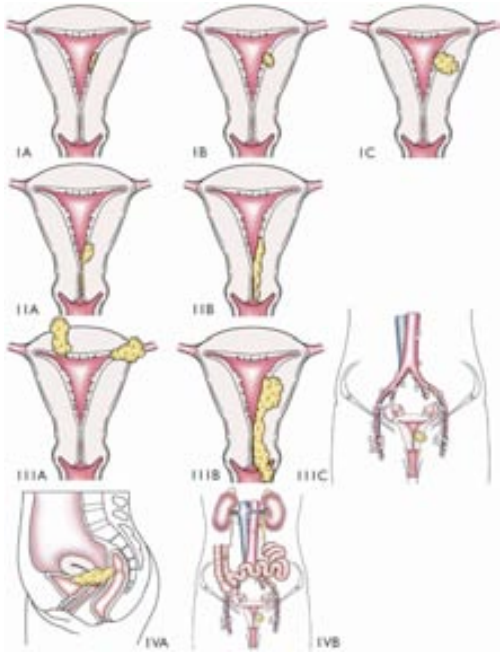


Figure 9. Definitions of the Clinical Stages in Carcinoma of the Corpus Uteri. A) FIGO nomenclature from 1988 [75]. **B)** Representative picture of FIGO Classification from 1988.

However, endometrial cancer staging was revised in 2009 and since chang data collection that allowed the identification and analysis of specific factors in surgical-pathological staging.

For instance, the Annual Report observed that there were no significant d between the five-year survival of stages Ia G1, Ib G1, Ia G2 and Ib G2 of en carcinoma. As a result, the main differences with the previous staging systen (1) stage I is no longer divided into a, b and c; instead, Ia= no myometrial in myometrial invasion <50% (previously Ia and Ib) and Ib= myometrial invas (previous Ic); and (2) stage II no longer has a subset a and b and it require stromal invasion. Involvement of the endocervical glandular portion of the now considered stage I.

Also, pelvic and paraaortic node involvements are no longer combined i substage, since data suggest that the prognosis is worse when the paraaortic involved. As a result, Stage IIIc is now categorized as IIIc1 (indicating posit nodes) and IIIc2 (indicating positive paraaortic nodes with or without posit nodes) [75, 76, 78, 79].

Table 4. Definitions of the Clinical Stages in Carcinoma of the Corpus U nomenclature from 2009.

Stage Ia*	Tumour limited to the endometrium or invasion of less than myometrium
Stage Ib*	Invasion equal to or more than half of the myometrium with/without glandular involvement
Stage II*	Cervical stromal invasion
Stage IIIa*	Tumour invades the serosa of the corpus uteri and/or adnexa an cytological findings
Stage IIIb*	Vaginal metastases
Stage IIIc*	
Stage IIIc1*	Metastases to pelvic lymph nodes
Stage IIIc2*	Metastases to paraaortic lymph nodes
Stage Iva*	Tumour invasion of bladder and/or bowel mucosa
Stage IVb*	Distant metastases, including intra-abdominal metastases and/or ing nodes

* Either G1, G2, G3

2.7 Prognostic Factors

The most important prognostic features in endometrial cancer are the surgical FIGO stages, myometrial invasion, histological type and differentiation grade; most are independent of each other [80]. Whether the 5-15% of patients with positive peritoneal cytology in the absence of extrauterine disease also classified as having stage IIIa lesions have a different outcome from those with a negative cytology remains controversial [81, 82]. Apart from this uncertainty, the FIGO stage reflects 5-year survival rates, which vary according to different series, but are around 81% for stage I, 69% for stage II, 51% for stage III, and 16% for stage IV [83-85].

Table 5. 5-year survival table according to FIGO stages by the American Cancer Society [86].

Stage	5-years survival
Stage Ia	88%
Stage Ib	75%
Stage II	69%
Stage IIIa	58%
Stage IIIb	50%
Stage IIIc	47%
Stage IVa	17%
Stage IVb	15%

The 5-year survival for the FIGO surgical stages based on the depth of myometrial invasion is further affected by the tumour grade, ranging from 95% for low-grade stage Ia lesions to only 42% for high grade stage Ib endometrial cancers [72, 85, 87]. The distance from the serosa might be a better prognostic factor than myometrial invasion from the cavity [88]. Vascular space invasion, although associated with tumour grade and depth of myometrial invasion, was an independent prognostic factor in some studies [89]. Vascular space invasion is present in about 37% of endometrial cancers and more than one vascular cross-section should be involved for it to be a prognostic indicator.

Non-endometrioid endometrial cancers such as serous and clear-cell carcinomas make up only about 10% of all endometrial cancers, but they account for more than 50% of the total of recurrences and deaths [71, 73, 90, 91].

2.8 Endometrial cancer treatment

The treatment of endometrial cancer is mainly surgical. In the higher stages, surgery is combined with chemo- and radiotherapy. The treatment of young patients with endometrial cancer is specially challenging, since some of these women might be nulliparous and have a wish to conceive [78].
Laparoscopic and robotic techniques have recently been proposed. In a study carried out in 2009, laparoscopic surgery in EC was associated with lower postoperative morbidity and shorter hospital stays when compared with the standard open surgical approach [92, 93].

The surgical procedure includes obtaining peritoneal fluid or washings for cytology, a total hysterectomy including the uterine cervix and a bilateral salpingo-oophorectomy, the surgical removal of the Fallopian tubes and the ovaries. In selected cases, an omentectomy (removal of part or all the *omentum*, a fold of the peritoneum, the thin tissue lining the abdomen that surrounds the stomach and other abdominal organs) and a thorough retroperitoneal lymph-node dissection are required. Manipulation of the tumour, including macroscopically involved lymph nodes, should be avoided to prevent the rare occurrence of port-site metastases [43].

Type II endometrial cancers require different surgical procedures because the pattern of spread is different from that in type I, with a higher likelihood of extrauterine disease. Given the propensity for lymphatic spread, a thorough lymphatic dissection is recommended in women with type II tumours [73, 94, 95]. The surgical management includes a midline abdominal skin incision, peritoneal biopsy samples, total hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and the biopsy of any suspect lesions [73].

Radiotherapy is generally an adjuvant therapy, indicated for those patients with a high risk of recurrence related to the grade and depth of myometrial invasion [40, 96]. Radiation can be delivered externally to the pelvis, as vaginal brachytherapy, or a combination of both. The goal of adjuvant radiotherapy is to treat the microscopic

disease that might be contained in the pelvic lymph-node regions and the central pelvic region, including the upper vagina [43].

Table 6. Summary of definitions of treatments [75].

Treatment	Definition
None	No treatment
Surgery alone	Surgery as first therapy and no other therapy (ies) within 90 days from the date of surgery. Subsequently, patients can be given any further treatment.
Radiotherapy alone	External radiotherapy and/or intracavitary irradiation as first therapy (ies) and no other therapy(ies) within 90 days from the end of teletherapy /brachytherapy. Subsequently, patients can be given any further treatment.
Radio-surgery	Intracavitary irradiation and/or external radiotherapy as first therapy(ies) and then surgery within 60 days from the end of brachytherapy/teletherapy. Subsequently, patients can be given any further treatment.
Surgery + adjuvant radiotherapy	Surgery as first therapy and then external radiotherapy and/or intracavitary irradiation within 90 days from the date of surgery. Subsequently, patients can be given any further treatment.
Surgery + adjuvant chemotherapy	Surgery as first therapy and then chemotherapy within 90 days from the date of surgery.
Adjuvant hormonal therapy	Surgery or radiotherapy or chemo-radiotherapy as first therapy and then hormonal therapy within 90 days from the end of surgery/radiotherapy/chemo-radiotherapy. Subsequently, patients can be given any further treatment.

In surgical FIGO stage I–II type 1 or 2 endometrial cancer, there is currently no proof that adjuvant hormone therapy or chemotherapy results in a better outcome [43]. However, pilot studies showed improved outcomes after the combination of both local and systemic treatment in early-stage carcinosarcoma and type II endometrial cancer, though this finding must be confirmed [97-99].

According to the SEGO (Spanish Society for Obstetrics and Gynaecology) guidelines for Type I endometrial cancer, sequential treatment with chemotherapy (Box 1) is recommended after adjuvant radiotherapy only in the advanced clinical stages, because they are directly related to an increased risk of recurrence. The

pharmacological regimen of choice for Type I endometrial cancer consists of 4 cycles of carboplatin and paclitaxel every 21 days. Alternatively, adriamicyn and cisplatin may be administrated every 21 days [100].

Type II endometrial carcinoma, like serous tumours and clear cell carcinoma, has a high risk of recurrence and therefore chemotherapy is the treatment of choice. The first-line pharmacological regimen for Type II endometrial cancer consists of 4 to 6 cycles of carboplatin and paclitaxel followed by radiotherapy with or without brachyteraphy [100].

In 1961, Kelly and Baker studied the possibility to treat patients with advanced endometrial cancer with a progestational agent after the role of estrogen in the aetiology of endometrial carcinoma and the profound effect of progesterone on the normal endometrium were described [101].

The most commonly used progestational agents are 17-hydroxyprogesterone caproate, medroxyprogesterone and megestrol acetate. However, the optimum dose for progestational treatment has not yet been determined. Moreover, the first treatments with progestational regimens were applied only to the most advanced or recurrent diseases [77].

Concerning progestational therapy, the following generalizations can be made: (1) the response rate for patients with advanced or recurrent endometrial carcinoma ranges from 10 to 30%, probably in relation to receptor levels in the tumour; (2) well-differentiated cancers respond best; (3) progesterone receptor levels decrease sharply as the grade of the tumour increases; (4) clinical responses may not occur before 7 to 12 weeks of therapy; (5) two-thirds of patients will not respond; (6) there is no published evidence that progestational agents employed as adjuvants offer any benefit [77].

Hormonal therapy by agents other than progestagens has been extensively studied. Extrapolating from the experience with breast cancer, investigators have used tamoxifen in doses of 20 to 40 mg daily for patients with advanced or recurrent endometrial carcinoma. Not unlike the progestin experience, it would seem that tamoxifen is more likely to be effective in patients with low-grade tumours, receptor positivity, and either no previous hormone therapy or a prior response to progestin therapy [77].

OX1. Definition from the NCI Drug Dictionary of the drugs used for the tratment of EC.

arboplatin, or cis-Diammine (1,1-cyclobutanedicarboxylato). A second-generation platinum compound vith a broad spectrum of antineoplastic properties. Carboplatin contains a platinum atom complexed with wo ammonia groups and a cyclobutane-dicarboxyl residue. This agent is activated intracellularly to form eactive platinum complexes that bind to nucleophilic groups such as GC-rich sites in DNA, thereby inducing ıtrastrand and interstrand DNA cross-links, as well as DNA-protein cross-links. These carboplatin-induced NA and protein effects result in apoptosis and cell growth inhibition. This agent possesses tumouricidal ctivity similar to that of its parent compound, cisplatin, but is more stable and less toxic.

aclitaxel is a compound extracted from the Pacific yew tree *Taxus brevifolia* with antineoplastic activity. aclitaxel binds to tubulin and inhibits the disassembly of microtubules, thereby resulting in the inhibition of ell division. This agent also induces apoptosis by binding to and blocking the function of the apoptosis ıhibitor protein Bcl-2 (B-cell Leukemia 2).

oxorubicin; trade name **Adriamycin**; also known as hydroxydaunorubicin, The hydrochloride salt of oxorubicin, an anthracycline antibiotic with antineoplastic activity. Doxorubicin, isolated from the acterium *Streptomyces peucetius var. caesius*, is the hydroxylated congener of daunorubicin. Doxorubicin ıtercalates between base pairs in the DNA helix, thereby preventing DNA replication and ultimately ıhibiting protein synthesis. Additionally, doxorubicin inhibits topoisomerase II which results in an ıcreased and stabilized cleavable enzyme-DNA linked complex during DNA replication and subsequently revents the ligation of the nucleotide strand after double-strand breakage. Doxorubicin also forms oxygen ee radicals resulting in cytotoxicity secondary to lipid peroxidation of cell membrane lipids; the formation f oxygen free radicals also contributes to the toxicity of the anthracycline antibiotics, namely the cardiac nd cutaneous vascular effects.

isplatin, cisplatinum, or *cis*-diamminedichloroplatinum. An inorganic platinum agent (cis-iamminedichloroplatinum) with antineoplastic activity. Cisplatin forms highly reactive, charged, platinum omplexes which bind to nucleophilic groups such as GC-rich sites in DNA, inducing intrastrand and ıterstrand DNA cross-links, as well as DNA-protein cross-links. These cross-links result in apoptosis and ell growth inhibition.

3 Molecular bases of endometrial carcinoma

3.1 Dualistic model for endometrial carcinogenesis and molecular genetics

In 1983, Bokhman [102] suggested that there were two pathogenetic forms of endometrial adenocarcinoma: the first presented in women with obesity, hyperlipidemia, and signs of hyperestrogenism such as anovulatory bleeding, infertility, late onset menopause, hyperplasia of ovarian stroma and endometrial hyperplasia; the second presented in women without these signs [65]. A decade later, molecular studies gave support to this model. Currently, endometrioid and serous carcinoma, which represent the main phenotypes of type I and II endometrial carcinomas, respectively, are characterized by distinctive types of genetic instability and molecular alterations [103].

3.1.1 Type I – Endometrioid Endometrial Carcinoma

Type I endometrial carcinoma or oestrogen-dependent endometrioid endometrial carcinoma (EECs) is an oestrogen-related tumour. These tumours are low grade, low stage (confined to the uterus), maintain the endometrioid morphology and are frequently associated with hyperplasia, in particular with atypical hyperplasia, and are characterized by a favourable prognosis. The driving force behind this group of tumours is unopposed estrogenic stimulation [64]. The rare mucinous adenocarcinomas are also considered type I carcinomas, since they usually express ER (Estrogen Receptors) and/or PR (Progesterone Receptors) and are of low histopathologic grade [103].

Endometrioid is the most common type of endometrial carcinoma and amounts to approximately 80% of cases; consequently, it has been studied more extensively than type II carcinoma. In addition, its association with hyperplastic precursor lesions has facilitated the identification of relatively early events [104].

Molecular genetics associated with Type I or EEC:

PTEN

Inactivation of the *PTEN* tumour-suppressor gene (formerly known as MMAC1) is the most common genetic defect observed in endometrioid carcinoma and is reported in up to 83% of tumours preceded by a histologically discrete premalignant phase [105]. Mutations have been detected in approximately 20% hyperplastic lesions, both with and without atypia, suggesting that mutations in *PTEN* occur relatively early in the pathogenesis of endometrioid carcinoma [106, 107].

PTEN is a tumour suppressor gene encoding a lipid phosphatase which acts to maintain G1 arrest and to enable apoptosis through an AKT-dependent mechanism [108, 109]. The downstream targets of phosphorylated AKT include a number of molecules that directly affect cell cycle regulation and apoptosis. Thus, loss of PTEN function results in the ability of cells to proliferate and escape cellular senescence. There is some evidence that the protein focal adhesion kinase is also a PTEN substrate, and alterations in its function may play a role in cell motility [104].

PTEN acts in opposition to phosphotidylinositol-3-kinase (PIK3CA) to control levels of phosphorylated AKT. The cooperative effect between these two elements results in the promotion of neoplastic transformation [110].

PTEN inactivation may be caused by a variety of mechanisms. The most commonly observed *PTEN* defect is inactivation of both alleles to generate a protein null-, or complete loss of function- phenotype. Mutations or deletions resulting in loss of heterozygosity (LOH) in chromosome 10q23 are detected in 37% to 61% of cancers [111, 112]. Promoter methylation has been postulated as an alternative transcriptional inactivating event [113].

Inactivation of *PTEN* caused by mutation is associated with an early stage and favourable survival. The 5-year survival rate in those with mutations is about 80%, in contrast with 50% in those lacking mutations [114].

Microsatellite instability (MSI)

Approximately 20% of sporadic endometrioid endometrial cancers of all grades demonstrate a molecular phenotype referred to as MSI [115-118]. MSI is defined as alterations in the length of short repetitive DNA sequences. The instability of the repeats is a direct consequence of the lack of intact DNA mismatch repair, an essential system for correcting DNA sequence errors created during replication. In endometrioid carcinoma, and other tumours, the DNA mismatch repair system is disabled either through intragenic mutation of one of the DNA mismatch repair genes or, more commonly, via promoter hypermethylation of the *hMLH1* gene [119]. The absence of DNA mismatch repair results in an increase in the rate of mutation in other cancer-causing genes, thus accelerating tumourigenesis.

Therefore, MSI is due to inactivation of any of a number of intranuclear proteins that comprise the mismatch repair system, leading to accumulation of structural changes in coding and non-coding repetitive elements of many genes [120]. *MLH1* inactivation, a component of the mismatch repair system, is the most common mechanism in the endometrium and is accomplished by hypermethylation of CpG islands in the gene promoter region, a process known as epigenetic silencing [119]. Inherited or somatically acquired mutations of *MSH6*, another mismatch repair element, are also common in patients with MSI endometrial cancers [121]. A single nucleotide insertion (frameshift) mutation in *MSH3* has been described less frequently in MSI endometrial cancers.

MSI due to abnormal methylation of *MLH1* is an early event in endometrial carcinogenesis that has been described in precancerous lesions [106, 115, 122]. The 5-year survival rate of patients with MSI is 77%, in contrast with 48% of negative MSI cases [123].

K-Ras

K-Ras encodes for a small inner plasma cellular membrane GTPase protein of 21 kDa that has a central role in the regulation of cell growth and differentiation by transducing signals from activated transmembrane receptors.

Mutations in *K-Ras* result in constitutive activity, even in the absence of an activated receptor, and have consistently been identified in 10–30% endometrial cancers in several studies. The mutations have been found in all grades of endometrioid carcinoma and have been reported in complex atypical hyperplasia, suggesting a relatively early role for *K-Ras* mutations in this tumour type [124-126]. There is a higher frequency of *K-Ras* mutations in MSI cancers [127].

β-catenin *(CTNNB1)*

Gain of function mutations in exon 3 of the *CTNNB1* gene at 3p21 are seen in 25% to 38% of type I cancers [128-130]. These mutations in exon 3 result in stabilization of the protein, cytoplasmic and nuclear accumulation, and participation in signal transduction and transcriptional activation through the formation of complexes with DNA-binding proteins [131]. β-catenin is a component of the E-cadherin-catenin unit, essential for cell differentiation and maintenance of normal tissue architecture, which plays an important role in signal transduction. In addition, it is an important member of the signal transduction pathway Wnt required for adult tissue maintenance. Indeed, malfunction in Wnt signalling promotes human degenerative disease and cancer [132].

β-catenin mutations may represent a pathway to endometrial carcinogenesis characterized by squamous differentiation and independent of *PTEN*. Although MSI, *PTEN*, and *K-ras* mutations frequently coexist with each other, these molecular abnormalities are not usually seen in tumours with β-catenin alterations [131]. When abnormal, β-catenin expression changes are usually seen throughout all tumour cells. β-catenin changes are also present in some premalignant lesions suggesting that β-catenin mutation is an early step of endometrial tumourigenesis clonally represented in all tumour cells [133, 134]. Moreover, changes in β-catenin activity may also contribute to later tumour progression [135].

Other genes

The phenotype and behaviour of type I cancers are codetermined by cumulative genetic factors that can include mutations other than those mentioned above.

E-cadherin is a transmembrane protein with five extracellular domains and an

intracellular domain that connects to the actin cytoskeleton through a complex with the cytoplasmic catenin. Decreased E-cadherin expression is associated with loss of cell–cell cohesive forces and has been shown to precede tumour cell motility. Loss of E-cadherin is a hallmark of the epithelial to mesenchymal transition (EMT). EMT involves the loss of intercellular cohesion and the modification of the cytoskeleton, leading to increased motility and invasion [136]. There is substantial evidence that in EC the development of transition features from epithelial to mesenchymal may be associated with myometrial invasion, a determinant prognostic parameter [137]. These features include a decrease in cell polarity and cell-to-cell contact, remodelling of the cytoskeleton, migratory phenotype and a mesenchymal-like gene expression program. Under such circumstances, cells show the increased expression of some genes, such as Snail, Twist, Slug and *HMGA2*, as well as a decreased expression of E-cadherin. Down-regulation of E-cadherin acts as a main player of epithelial to mesenchymal transition, and modifies other molecules involved in cell-cell contacts. Down-regulation of E-cadherin also renders cells with a migratory phenotype [138]. Decreased expression of E-cadherin is found in about 5-40% endometrioid carcinomas [139, 140].

p53 mutations are found in a subset of approximately 10–20% endometrioid carcinomas, mostly grade 3 [125]. *p53* is a tumour suppressor gene that prevents the propagation of cells with DNA damage. After DNA damage, the p53 protein accumulates in the nucleus and provokes cell cycle arrest by inhibiting cyclin-D1 phosphorylation of the Rb gene, thereby promoting apoptosis [141]. The apoptotic index and p53 nuclear accumulation have been shown to be independent predictors of recurrence and short survival [142].

HER2/NEU oncogene codes for a transmembrane receptor tyrosine kinase involved in cell signalling. Overexpression of *HER2/NEU* seems to play a role in 10–30% of grades 2 and 3 endometrioid adenocarcinomas [143, 144]. Mutations in *p53* and the amplification and overexpression of *HER2/NEU* characterize late events during progression and the dedifferentiation of endometrioid carcinoma [125].

ETV5/ERM (Ets-related protein) is a transcription factor of the ETS family and a divergent member of the winged helix-turn-helix super-family. ETV5 binds to

sequences containing the consensus pentanucleotide 5'-CGGA(AT)- 3'. ETV5 is a proto-oncogene that plays a role in the progression of breast cancer, functions as an adaptor molecule in the interactions of adhesion receptors and intracellular tyrosine kinases, and is required for spermatogonial stem cell self-renewal [145, 146].
It has been postulated that ETV5 intervenes during the early events of endometrial tumourigenesis and is associated with an initial switch to myometrial infiltration. This upregulation correlates with the process of tumourigenesis, from normal atrophic endometrium to simple and complex hyperplasia and on to carcinoma [147, 148].

Steroid receptor genes

Estrogens and progestins act reciprocally on the hormonally responsive endometrial tissue to modify endometrial cancer risk. Progestins have the ability to oppose the biologic effects of coexisting estrogens through down-regulation of the estrogen receptor itself and consequently the biologic effects of admixtures of circulating progestins and estrogens are dominated by the progestational component. Women exposed to estrogens without the opposing effects of progestins show a dose and duration dependant 2- to 10-fold increased cancer risk [149].

There are several postulated mechanisms by which sex hormones affect endometrial cancer risk, and it is likely that all are relevant to varying degrees. Estrogen promotes cell proliferation and inhibits apoptosis through a complex downstream cascade of transcriptional changes that may include modulation of tumour suppressor function, for instance the modulation of *PTEN* [150].

3.1.2 Type II – Non-Endometrioid Endometrial Carcinoma

The second group, Type II or non-endometrioid endometrial carcinomas (NEECs), is comprised of high-grade papillary, serous and clear cell carcinomas. These tumours affect relatively older women and are not usually preceded by a history of unopposed estrogen exposure, but rather by an atrophic endometrium. NEECs are frequently associated with endometrial intraepithelial carcinoma. Type II tumours have an aggressive clinical course, a greater propensity for early spreading, and a worse prognosis than the more common endometrioid adenocarcinomas [151].

Molecular genetics associated with non-EEC:

p53

There have been fewer studies on serous carcinoma, largely owing to its relatively low frequency, which accounts for only 10–15% of all endometrial carcinomas. The *p53* tumour suppressor gene is altered in a significant number of cases. Approximately 75% EICs (Endometrial Intraepithelial Carcinoma), the putative precursor of serous carcinoma, have mutations in *p53*, suggesting a role for its inactivation early in the development of this aggressive tumour type [152].

This contrasts with endometrioid carcinoma, in which *p53* mutations are less common and are largely confined to grade 3 tumours [125]. Thus, it is possible that the mutation of *p53* early in the pathogenesis of serous carcinoma is an important factor in determining its aggressive behaviour. In addition, the fact that *p53* mutations occur most commonly in grade 3 endometrioid and serous carcinomas may provide an explanation for overexpression and mutation of *p53* as an independent indicator of poor prognosis [153].

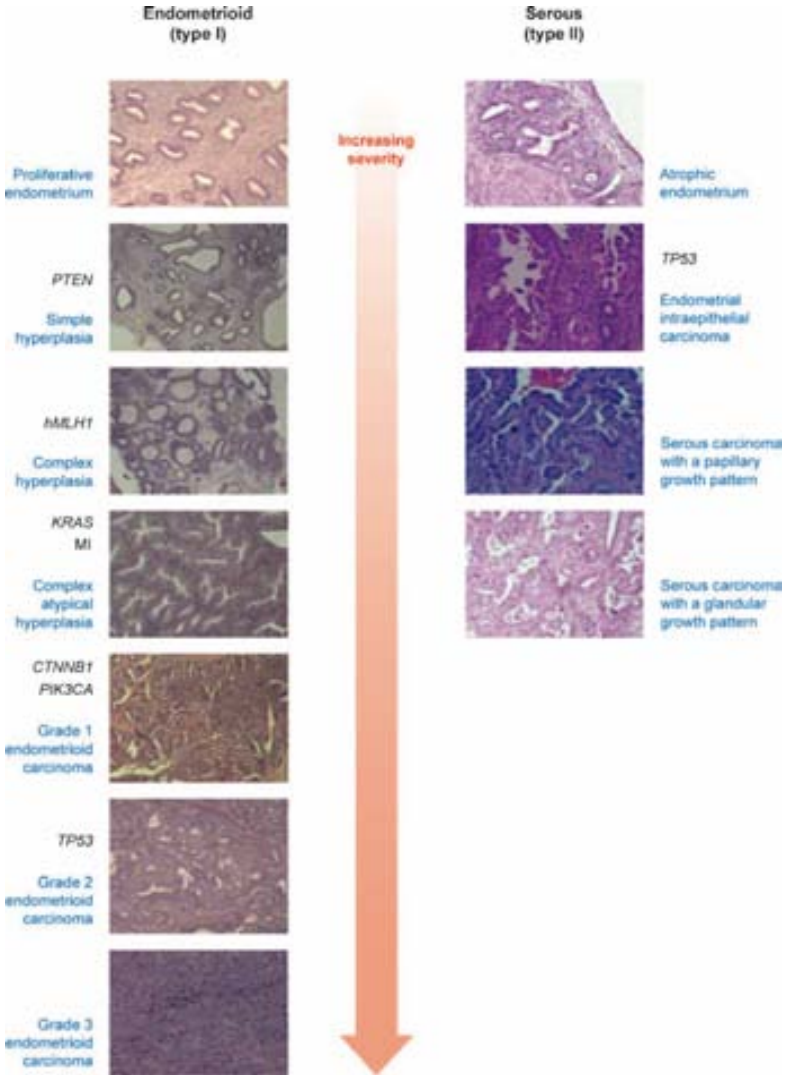
HER2/NEU

Its overexpression and gene amplification were found in about 45% and 70% of serous carcinomas, respectively [154, 155].

See next page;

Figure 10. Left: **Table of molecular differences between Endometrioid and Non-Endometrioid Subtypes of Endometrial Adenocarcinoma** (EIN: Endometrial Intraepithelial Neoplasia, EGD: Endometrial Glandular Dysplasia, EIC: Endometrial Intraepithelial Carcinoma). Modified from *Molecular Pathology of Gynecologic Cancer* [65]. Right: **Figure of the histological and molecular genetic progression of endometrial carcinoma**. The molecular genetic alterations are indicated at the earliest point of identification in the progression [104].

Feature	Type I	Type II
Incidence	80%	</ = 20%
Age	Pre-/perimenopausal	> 60 yr
Cell differentiation	Low grade	High grade
Alternated designation	Endometrioid	Non-endometrioid
Histology	Endometrial, mucinous, adenosquamous, secretory	Papillary serous, clear cell, carcinosarcoma
Grades	1-3	Not applicable
Behaviour	Indolent	Aggressive
Risk Factors	Endocrine (unopposed estrogen)	Unknown
Precursor lesion	EIN	??EGD ?? EIC
p53 mutation	5-10%	80-90%
PTEN inactivation	55%	11%
MLH1 inactivation	17%	5%
□-Catenin inactivation	25-38%	Rare
K-ras inactivation	13-26%	0-10%
Loss of estrogen and progesterone receptors	27-30%	76-81%



4 Endometrial cancer dissemination

Disease limited to the uterus is a favourable prognostic factor. However, approximately 1 in every 3 women who die of EC were considered to have early locoregional disease at the time of the primary treatment [8]. Most treatment failures and the accompanying compromised longevity probably result from the inability to recognize occult extrauterine spread.

EC has 4 pathways of dissemination that can present alone or in combination:

1. **Contiguous** (mainly to the vagina)

Histological grade 3 and lymphovascular space invasion are proven predictors of vaginal relapse in stage I EC [156]. Although vaginal recurrences can be successfully salvaged in a great proportion of cases [157], the addition of vaginal brachyterapy to the initial surgical treatment can significantly reduce the risk of such recurrence [158].

2. **Lymphatic**

Lymphatic invasion is more likely to occur when cervical stroma involvement and positive lymph nodes are present [82].

3. **Haematogenous**

Deep myometrial invasion is the strongest predictor of haematogenous recurrence [82].

4. **Peritoneal**

Predictors of peritoneal relapse are:

- Stage IV disease
- Stage II-III disease with 2 or more of the following risk factors: cervical invasion, peritoneal cytological results positive for EC, positive lymph nodes and nonendometrioid histological findings [82].

4.1 Myometrial invasion

The depth of myometrial penetration is a very important independent prognostic factor in stage I. Deeper penetration is associated with a higher probability of tumour recurrence and death [84, 159-161]. While an increasing depth of invasion is positively correlated with tumour grade, depth appears to be a more significant

prognostic factor and predicts the presence of extrauterine disease as detected during the surgical staging procedure. Moreover, deep myometrial invasion correlates with more undifferentiated tumours, lymphovascular invasion, node affectation and decreased global survival [138].

Only 1% of patients with disease confined to the endometrium have extrauterine disease as compared with patients with deep muscle invasion, where the incidence of pelvic node invasion rises to 17% and paraaortic nodal involvement rises to 25% [162]. DiSaia and colleagues found that patients with only endometrial involvement had an 8% recurrence rate, compared with 12% when there was superficial or intermediate myometrial invasion, versus 46% if there was involvement of the outer third of the myometrium [160].

The molecular pathology of myometrial infiltration that defines the initial steps of invasion in endometrial cancer is based on alterations in different genes, with the down regulation of E-cadherin as a main player of the epithelial to mesenchymal transition, as well as modifications on other molecules involved in cell-cell contacts such as β-catenin, which confers a migratory phenotype to cells. As a result of these modifications, cells acquire a migratory and invasive phenotype.

Our group has reported that altered signalling pathways and transcription factors such as *ETV5* or *RUNX1* are associated with myometrial invasion, histological grade and metastasis [138].

5 *ETV5* Transcription Factor and its involvement in endometrial carcinoma

5.1 ETS Family: PEA3 group

The ETS genes encode a family of transcription factors of about 30 members. In most cases these are transcriptional activators, and more rarely transcriptional repressors. These transcription factors share a preserved 85-amino-acid motif, the ETS-domain, which is the signature of the family [163]. This domain enables the transcription factors to bind to a 9- nucleotide DNA sequence with the central consensus core 5′-GGAA/T-3′: the ETS Binding Site (EBS) [164]. The sequences flanking this central

motif determine the binding specificity for each ETS family member [165, 166]. Systematic analysis of the DNA sequence specificity revealed that the PEA3 family members preferentially bind to 5′-ACCGGAAGT-3′. Consequently, the importance of bases in determining the strength of DNA-binding diminishes with the distance from the 5′-GGAA-3′ core [167]. As activation domains, the ETS domain is regulated intramolecularly: amino acids on both sides of the ETS domain inhibit its DNA-binding ability [168-171].

Phylogenetically, the members of this transcription factor family are classified in groups according to the sequence of the ETS-domain, the position of this domain in the protein, and the presence of other specific conserved functional domains. The 13 identified groups are: ETS, TEL, YAN, SPI, ERG, PEA3, ELF, DETS4, ELK, GABP, ER71, ERF, and ESE [172].

The PEA3 group is composed of the following: three members, *ETV5* (also called *ERM*), *ETV1* (also called *Er81*), and *ETV4* (also called *E1Af* or *PEA3*), which share the highly conserved ETS domain; and two conserved transactivating domains, one located at the amino-terminus domain (AD) and the other at the carboxy-terminus (Ct), which are able to synergize [168, 169, 173] (Figure 11). Recent data on ETV5 and ETV4 suggest that the Ct domain also plays a role in the stability of the protein [174, 175].

Like many transcription factors, the PEA3 group members undergo post-translational modifications that regulate their transactivation capacity. The most common modification found in the PEA3 group of proteins is phosphorylation, as they are targets of the MAPK pathway including Ras, Raf-1, MEK, ERK-1, and ERK-2. Phosphorylation of specific serine and threonine residues generally increases the transactivation capacity of the PEA3 group member [176-179]. Moreover, ETV5 and ETV1 are also phosphorylated through the PKA-mediated pathway [170, 176, 180, 181]. Post-translational modifications of lysines also play crucial roles in the regulation of transcription, generally on histone proteins but also on transcription factors. ETV5 and ETV4 are also conjugated to ubiquitin and degraded via the

ubiquitin–proteasome pathway, which probably regulates the activity of these transcription factors [174, 175].

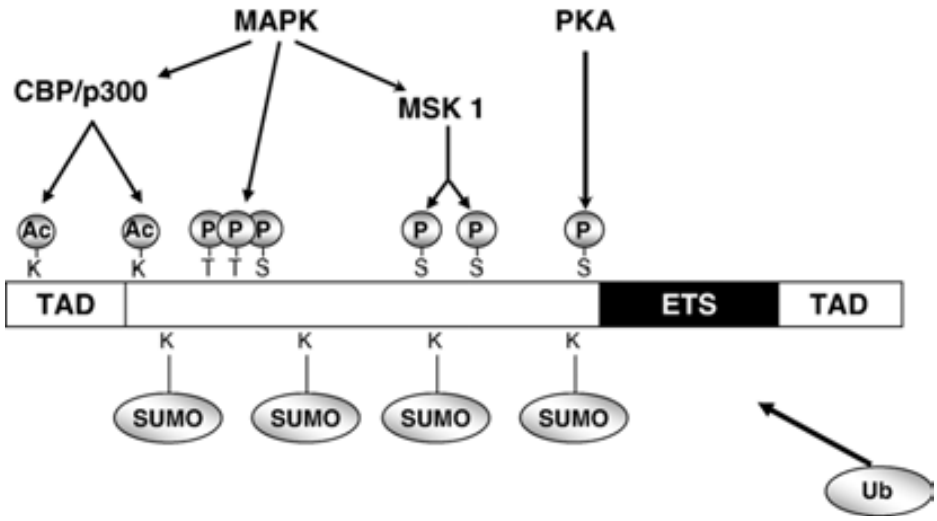


Figure 11. Representation of a generic PEA3 group prototype with its different functional domains and regulating pathways [182].

5.2 Physiological roles of *ETV5*

Analysis of *ETV1*, *ETV4* and *ETV5* mRNA levels revealed that their genes are expressed in numerous organs both during embryonic development and in adults [183-185].

One emerging role of PEA3 factors is in branching morphogenesis, where primitive epithelial buds bifurcate to generate tree-like ducts or acinar structures. This process also involves the adjacent mesenchyme, which interacts with the epithelial cells in complex ways. For instance, both *ETV4* and *ETV5* are expressed at the tip of the epithelial ducts during mesonephric differentiation in the mouse embryo, while *ETV1* is expressed at low levels in the adjoining mesenchyme. After differentiation of the renal glomeruli, *ETV4* and *ETV5* expression sharply decreases and only remains at sites of poorly differentiated metanephrogenic cap tissue [186]. These data suggest that *ETV4* and *ETV5* are especially involved in kidney development and perform similar functions.

Also, *ETV5* is required for the self-renewal of spermatogonial stem cells [146, 187] and it influences the development competence of germ cells [188]. Some studies using model mice demonstrated that ETV5+/− males were fertile, but that adult ETV5−/− males were sterile. Microarray analysis of primary Sertoli cells from *ETV5*-deficient mice showed alterations in secreted factors known to regulate the hematopoietic stem cell niche [189]. A recent study demonstrated that ETV5 is expressed in germ cells in neonates, suggesting a role in germ cell functions [190]. It has been recently shown that ETV5 regulates expression of chemokines that are involved in the migration of spermatogonial stem cells [191]. The role of *ETV5* in female fertility is well illustrated in studies using ETV5-deficient mice, where it has been implicated in implantation as well as in ovarian functions [192]. In a recent study, ETV5−/− female mice showed complex ovarian defects that manifested as reduced developmental competence of oocytes after fertilization as well as mating and ovulation defects [188]. Defects in the ovarian tissue architecture were noted in early stages of the ovarian development. Ovulation was compromised in mature ETV5−/− mice even after gonadotropin treatmen and some embryos of ETV5−/− females showed compromised development. Collectively, these results suggest that *ETV5* is important for the developmental competence of germ cells and the regulation of responses to steroid hormones in mice.

ETV5 regulates the transcription of its target genes by associating with the basal transcription complex proteins TAFII60, TBP and TAFII40 [193]. It also interacts with the androgen receptor, which leads to the repression of Ets-mediated transactivation [194]. One of the AP1 complex proteins, c-Jun, also interacts physically with the Ct domain of ETV5 to synergistically enhance transcriptional activation [195]. Furthermore, ETV5 [196] interacts with the p300 transcriptional coactivator, an enzyme with histone acetyltransferase activity (HAT). More recently, the SUMO conjugating enzyme Ubc9 was also shown to interact with ETV5 [197].

5.3 ETV5 and cancer

Breast cancer

ETV5 becomes up regulated at mRNA level in mammary tumours which have *HER2/NEU* overexpressed, suggesting that it can be a downstream effector of the HER2/Neu oncoprotein [198, 199]. ETV5 regulates MMPs (Matrix Metallopeptidases) [200], which are involved in tumour cell migration and invasion. Accordingly, *ETV5* ablation suppresses proliferation, migration and tumour formation by MMT-060562 mouse mammary tumour cells [201]. Together with the observed upregulation of *ETV5* in breast cancer, these data suggests that ETV5 overexpression contributes to the causation of mammary tumours.

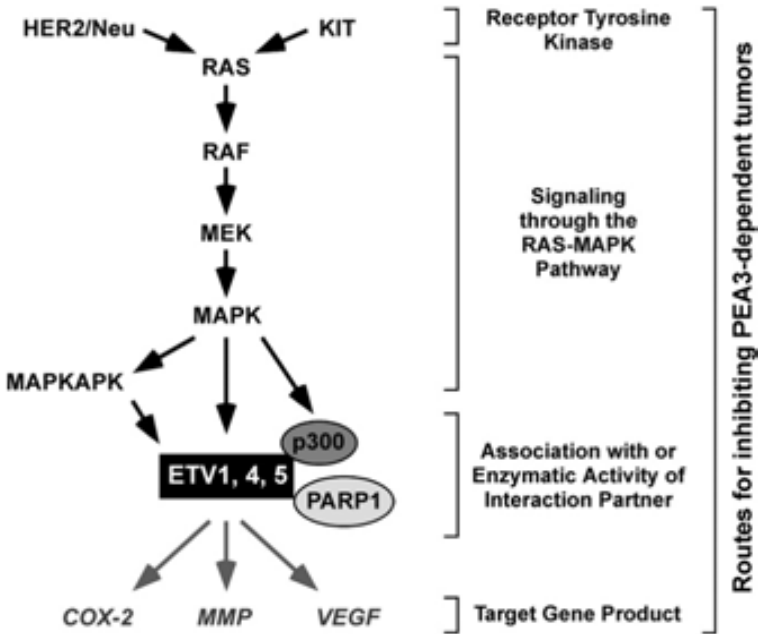


Figure 12. ETV5 pathway in breast cancer [202].

Ewing's sarcoma

Ewing's sarcoma is a rare, very aggressive primary bone tumour. There are approximately 200–300 cases annually in the US and it mostly affects children and adolescents. The defining feature of these tumours are chromosomal translocations involving the Ewing's sarcoma (*EWS*) gene (or in rare instances a homolog of *EWS*, *FUS*) and one out of five ETS genes: *FLI1, ERG, FEV, ETV1* or *ETV4* [203]. Even

though the principal translocations in Ewing's sarcoma are not produced by the *ETV5* factor, some studies suggest that overexpression of *ETV5* may be sufficient to induce Ewing tumours [204].

Prostate cancer

The majority of prostate tumours display chromosomal translocations involving the *TMPRSS2* gene, and it is described that *ETV5* can fuse to *TMPRSS2* in prostate tumours [205]. The consequence of such translocation is the generation of a *TMPRSS2-ETS* fusion gene, in which the expression of the ETS protein is controlled by the *TMPRSS2* gene promoter/enhancer. Since the *TMPRSS2* serine protease gene is androgen-inducible and highly expressed in the prostate [206], its translocations induce the androgen-dependent expression of ETS proteins in this organ, thus guaranteeing that ETS proteins become overexpressed in prostate tumours. While *TMPRSS2* is the most frequent translocation partner for ETS proteins in prostate tumours, other gene fusions that put ETS expression under the control of various androgen-dependent or constitutively active gene promoters were observed [207].

Ovarian cancer

Our group recently examined the role of the ETS transcription factor ETV5 in epithelial ovarian cancer and described *ETV5* as being upregulated in ovarian tumour samples when compared to ovarian tissue controls [208]. In ovarian cancer cells, we showed that ETV5 regulated the expression of cell adhesion molecules, enhancing ovarian cancer cell survival in anchorage-independent conditions and suggesting that it plays a role in ovarian cancer cell dissemination and metastases into the peritoneal cavity. We analyzed by gene expression microarray technology the genes whose expression was altered in an ovarian cancer cell line with a stable down-regulation of *ETV5*. The analysis of the genes and signalling pathways under the control of ETV5 in OV90 cells has unravelled new signalling pathways that interact with ETV5, among them the cell cycle progression and the TGFβ signalling pathway. We have also demonstrated that ETV5 can regulate the expression of FoxM1 oncogenic transcription factor in ovarian cancer cells [209].

5.4 ETV5 and endometrial carcinoma

During the past few years our group has investigated myometrial invasion in endometrial cancer. Microarray analysis of endometrioid endometrial carcinomas comparing healthy endometrium against EEC showed an overexpression of the *ETV5* transcription factor. This overexpression was restricted to myometrial invading tumours. Validation of microarray results by RTqPCR showed that ETV5 was overexpressed in stage Ic (currently Ib) endometrial carcinoma samples, a stage associated with myometrial invasion [147]. We have consistently described that ETV5 enhances migration of the human endometrial Hec1A cancer cell line *in vitro* and induces a more aggressive and infiltrative pattern of myometrial invasion. ETV5 function is mediated both *in vitro* and *in vivo* through the increased activity of the matrix metalloproteinase-2. The specific location of ETV5 and MMP2 at the invasive front of myometrial infiltrating human endometrial carcinomas further reinforced the hypothesis of a role for ETV5 in early endometrial dissemination [148]. We concluded that in EEC, ETV5 acts through MMP2's gelatinolytic activity to confer invasive capabilities and that ETV5 is associated with an initial switch towards myometrial infiltration.

We have also described ETV5 as a protective factor against oxidative stress induced by tumour growth in endometrial cancer cells. We have characterized the specific upregulation of the nuclear dehydrogenase/reductase Hep27 as well as its ERM/ETV5- dependent mitochondrial location in Hec1A cells. Functional studies showed a protective role of Hep 27 against apoptosis induced by oxidative stress.

Finally, our group has demonstrated a direct role of ETV5 on epithelial-to-mesenchymal transition (EMT). In particular, ETV5 modulated Zeb1 expression and E-cadherin repression leading to a complete reorganization of cell-cell and cell-substrate contacts. ETV5-promoted EMT resulted in the acquisition of migratory and invasive capabilities in endometrial cell lines. Furthermore, we identified the lipoma-preferred partner (LPP) protein as a regulatory partner of ETV5, acting as a sensor for extracellular signals promoting tumour invasion. In conclusion, we propose that ETV5's transcriptional regulation of the EMT process through crosstalk with the

tumour's surrounding microenvironment, is a main event in the initiation of EC invasion [137].

6 Nuclear Protein 1

6.1 Characteristics of NUPR1

NUPR1, also known as com-1 (candidate of metastases-1) or p8 is a small 8 KDa protein described firstly over a decade ago as being overexpressed in pancreatic acinar cells during the acute phase of pancreatitis in rats. NUPR1 was thought to be preferentially upregulated in response to cellular stress [210]. Shortly after, Ree et al. independently identified a novel candidate of metastasis-1 (com-1) gene that was significantly upregulated in metastases formed in the central nervous system upon injection of cancer cells originally isolated from micrometastases in the bone marrow of a breast cancer patient [211]. It was postulated that com-1 participates in intracellular signalling that facilitates tumour establishments in a secondary organ [211]. It was subsequently found that com-1 was the human counterpart of the rat's p8 described earlier [210].

Human NUPR1 encodes an 82 aminoacid polypeptide containing a canonical bipartite domain of positively charged amino acids typical of nuclear-targeting signals (NLS) [212] and a N-terminal PEST (Pro/Glu/Ser/Thr-rich) region, suggesting a regulation of NUPR1 protein levels by the ubiquitin/proteasome system. It is located in chromosome 16 position p11.2, a region frequently amplified in breast cancer [213].

NUPR1 has two known isoforms: the longer isoform "a" consist of 100 amino acids in length, whereas the shorter isoform "b" is comprised of 82 amino acids. The relevance of the 18 amino acid region absent in the small isoform is not clear.
Homology search in databases yielded no significant homology between NUPR1 and other proteins of known function. However, some of its biochemical properties are shared by members of the high mobility group of proteins (HMGs), in particular by the HMG-I/Y subfamily (also known as HMGA1) [214]. Members of the superfamily of high mobility group (HMG) proteins are considered architectural elements of

chromatin. It is now known that they belong to a network of dynamic chromatin proteins that move constantly around the chromatin fiber, thereby dynamically modulating DNA-dependent processes [215]. The localization of NUPR1 in the cell varies; NUPR1 was found to be nuclear when cells were growing at low density and was distributed in nucleus and cytoplasm in dense cultures [216].

All these data suggest that NUPR1 is a complex molecule with diverse physiological and biological functions.

6.2 NUPR1 biological functions

NUPR1 has been implicated in a wide range of biological functions, which are context-dependent, sometimes contradictory and as yet not completely elucidated.
Initially, NUPR1 was shown to be expressed in pancreatic islets and in some pancreatic β-cell lines and was found to be a physiological mediator of glucose induced pancreatic β-cell growth [217]. Also, the increase in *NUPR1* mRNA was essential for the ability of several tissues to tolerate stress induced by systemic lipopolysaccharide (LPS) treatment, not as a mediator of tissue insult but rather as an important component of the cellular stress management program [218].

NUPR1 protein levels were significantly increased in heart failure, and therapeutic intervention reduced NUPR1 [219]. Goruppi et al. demonstrated that NUPR1 is required for ET-1-induced (endothelin-1) mesangial cell hypertrophy in diabetic nephropathy. The initiating events in diabetic nephropathy are triggered by hyperglycaemia and, possibly, by advanced glycation end products. Subsequently, excess levels of vasoactive peptides (especially endothelin-1 (ET-1)) accumulate in the diabetic kidney, and there is some evidence that these peptides mediate many of the pathophysiological changes associated with diabetic renal disease. NUPR1 has been proposed as an excellent marker for diabetic mesangial cell hypertrophy [220] and cardiac hypertrophy.

NUPR1 binds the Jun-activating binding protein 1 (Jab1) to induce translocation of the p27 checkpoint regulator from nucleus to cytoplasm and its subsequent degradation.

As a result, cyclin-dependent kinases Cdk2 and Cdk4 are freed from p27-mediated inhibition, allowing cell cycle progression [221, 222].

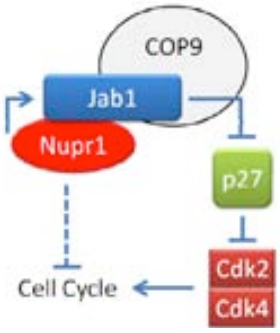


Figure 13. Cell cycle pathway with NUPR1 involvement [223].

In addition, NUPR1 regulates programmed cell death through its interaction with prothymosin-α. Cytochrome c is released from the mitochondria in response to pro-apoptotic stresses. Cytosolic cytochrome c binds to Apaf1 to form the apoptosome complex, which will induce the activation of pro-caspase 9 and the subsequent activation of pro-caspase 3, leading to cell death by apoptosis. The heptamerisation of the cytochrome c-Apaf1 complex to form the apoptosome is controlled by the NUPR1-prothymosin-α complex [222, 224].

Moreover, NUPR1 is involved in the ER-stress (Endoplasmic Reticulum) response. ER-stress increases levels of intracellular ceramide to induce NUPR1 expression, which in turn induces transcription of *ATF4*, *CHOP* and *TRB3*. ATF4 acts as a positive feedback of the pathway by inducing *NUPR1* gene activation. Activation of *TRB3* will induce autophagy by inhibiting the mTORC1 and Akt pathways (Figure 14) [225, 226].

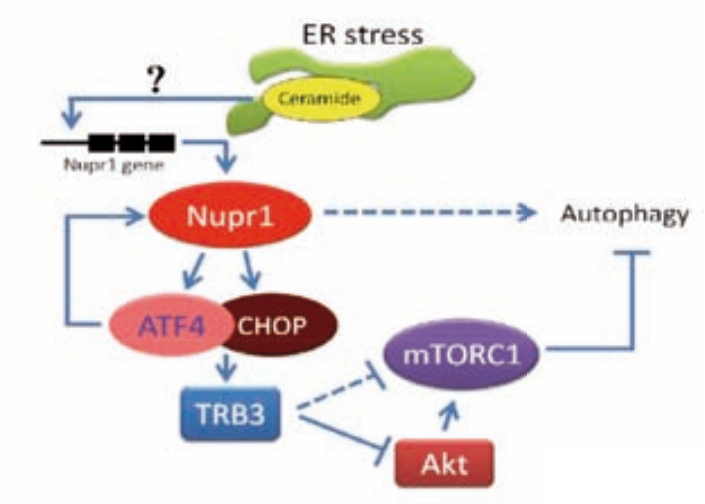


Figura 14. NUPR1 is involved in ER-stress.

Other reports describe that NUPR1 controls the expression of several target genes by regulating chromatin accessibility. NUPR1, by interacting with the *MSL* (male-specific lethal gene, a component of the histone acetyltransferase complex responsible for the majority of histone H4 acetylation implicated in the formation of higher-order chromatin structure complex), regulates histone acetylation/deacetylation and therefore chromatin accessibility and gene transcription. NUPR1 also interacts with the transcription factors PTIP and p300 to regulate the expression of several genes. It also interacts with p68, MyoD and p300 to regulate the expression of other genes, and by binding to p53, it is involved in the regulation of the cyclin-dependent kinase inhibitor p21 expression [227, 228].

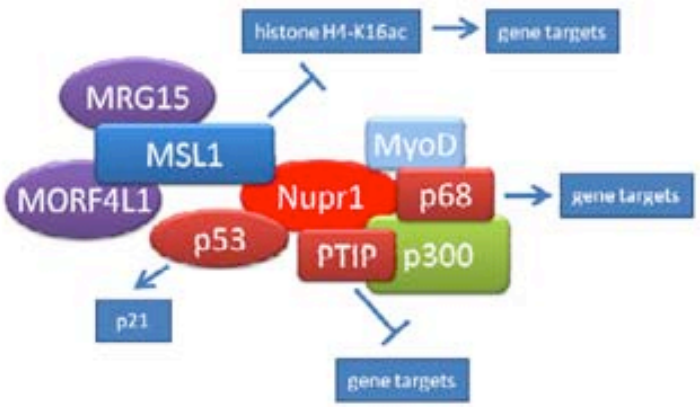


Figure 15. Proteins that interact with NUPR1 to modify chromatin accessibility [223].

Finally, NUPR1 is implicated in the TGFβ signalling pathway. This gene is a target of TGFβ through three Smad-specific sequences located on its promoter. TGFβ-induced cell cycle arrest requires NUPR1 expression. On the other hand, it improves the expression of some TGFβ target genes that favour tumour progression by enhancing the production of metalloproteases and, presumably, of EMT [219, 229-232].

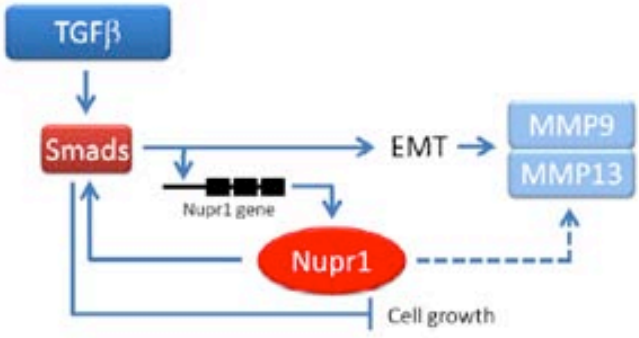


Figure 16. NUPR1 regulation via the TGFβ pathway [223].

6.3 NUPR1 and cancer

Various reports have shown NUPR1 to be both inducer and suppressor of cell and tumour growth. Moreover, *in vitro* results often disagree with the findings from *in vivo* studies. A possible explanation is that the microenvironment influences the activity of NUPR1.

NUPR1 as oncogene

NUPR1 has been described to help tumour cells to colonize metastatic sites in breast cancer during the early stages of the disease [211, 233]. Clark et al. reported that NUPR1 confers chemoresistance in breast cancer [234]. A recent study of 145 early-breast cancer (EBC) samples where the recurrently altered regions associated with a poor prognosis (*NUPR1* and *ERBB2*) were analyzed, suggests that the simultaneous gain of *NUPR1* and *ERBB2* can be a significant predictor of poor prognosis [235].

In pancreatic cancer, NUPR1 is overexpressed and contributes to mitogenic activity, but its inhibition sensitizes gemcitabine-resistant cells to treatment [236-238].

In brain tumour and brain metastases, an overexpression of mRNA levels of NUPR1 has been found [239]. NUPR1 is also frequently overexpressed in larger papillary carcinomas with node metastases. Ito et al. hypothesized that NUPR1 may be necessary for the development of papillary carcinoma in late stages [240].

Finally, in pituitary tumours, through NUPR1 mediation of the Cip/Kip family of proteins, NUPR1 maintains the tumourigenicity of transformed pituitary cells by

skipping cell cycle checkpoints and causing accumulation of genetic defects [241, 242].

Recent studies describe the role of NUPR1 in non-small cell lung cancer: downregulation of NUPR1 expression significantly inhibits non-small cell lung cancer H1299 cells proliferation, colony formation, arrest cells in G0 phase and increases apoptosis rate *in vitro*. Silencing of NUPR1 also suppressed tumour growth by tail vein injection of lentivirus encoded shRNA against NUPR1 *in vivo* [243].

NUPR1 as tumour suppressor

NUPR1 is expressed in lower levels in prostate cancer and its levels inversely correlate with invasiveness and tumour progression. It is reported that NUPR1 may act as a tumour suppressor in prostate cancer via its interaction with PGC-1 [244].

Similarly, Ishida et al. found that synovial sarcomas had a decreased expression of NUPR1 [245] and that restoring the expression of NUPR1 resulted in reduced proliferation and colony formation accompanied by increased apoptosis, suggesting that NUPR1 plays a growth inhibitory role in the growth of synovial sarcomas.

7 Nidogen 1

7.1 Characteristics of Nidogen 1

Nidogen, also known as entactin or enactin, is an ubiquitous basement membrane (BM) glycoprotein that consists of two amino (G1, G2) and one carboxyl (G3) globular domains that are connected by a rod domain composed primarily of EGF (epidermal growth factor-like) repeats. There are two closely related nidogen genes in mammals. Vertebrate biochemical studies show that the G3 domain binds with high affinity to the laminin-γ chain, while the G2 domain can bind perlecan and type IV collagen. In fact, nidogen-laminin binding has been characterised as one of the highest binding affinities known in nature [246]. The ability of nidogen to form a ternary complex with laminin and type IV collagen led to the suggestion that it may function as a link between BM (basement membrane) proteins [247]. Because of their wide range of binding partners, they have become considered as adapter proteins in this specialized extracellular matrix.

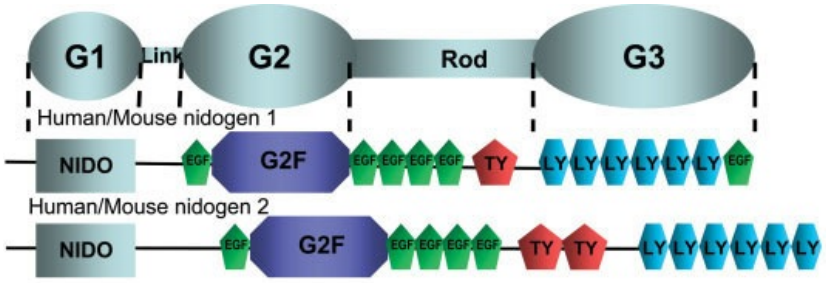


Figure 17. Representation of NID1 structure [248].

Entactin-1 was initially extracted from the extracellular matrix of differentiated mouse embryonal carcinoma cells, the same source as in Chung's et al. laboratory where laminin-111 had previously been isolated [249]. In independent studies, nidogen-1 was isolated from the murine Engelbreth- Holm-Swarm tumour, a transplantable sarcoma [250]. However, when sequence data became available, it became evident that this protein was identical and presumably the same as the 150 kDa protein previously immunoprecipitated with laminin antibodies in Chung's et al. laboratory [251].

Nidogens are produced by mesenchymal cells and fibroblasts and are mostly found in epithelial and endothelial basement membranes during development [252]. Chung et al. described that laminin and NID1 (Nidogen 1) are co-transported to the extracellular compartment, most likely as a pre-formed complex [253].

7.2 Nidogen1 biological functions

Nidogens have a wide repertoire of binding partners, in particular the other basement membrane components laminin, collagen IV, perlecan, and fibulin. The interactions of these components supposedly connect and stabilize the major networks of the basement membrane, and anchor the mesenchymal nidogen to the epithelial/endothelial basement membranes.

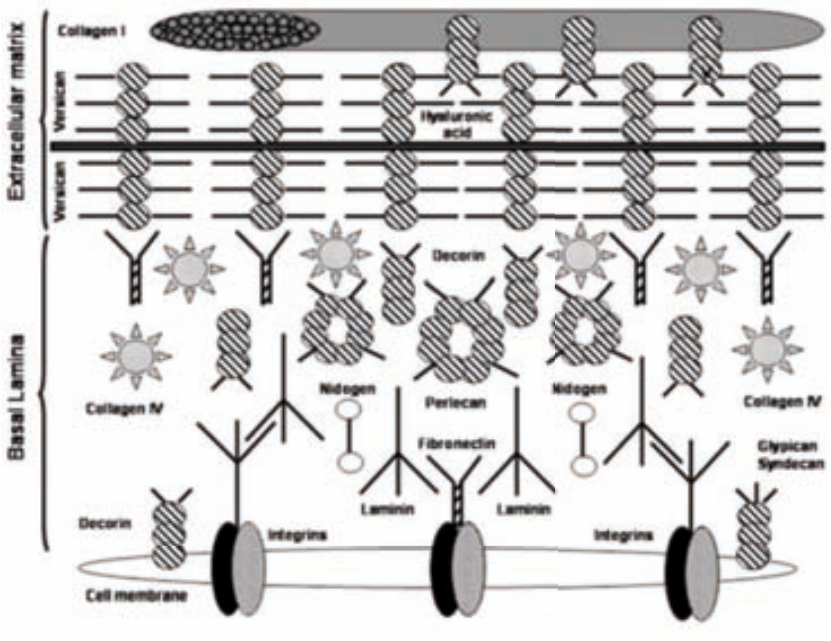


Figure 18. Representation of the structure of extracellular matrix and the basal lamina.

As aforementioned, nidogen is not generally a product of epithelia but of the adjacent mesenchyme, which led to the assumption that the binding of mesenchymal nidogen to epithelial laminin is a key event in epithelial formation. Some studies strongly suggested that laminin-nidogen binding is crucial to maintain nidogen in the extracellular matrix and that it is important in organ development [254-256].

The principal described biological functions of NID1 are:

(1) Extracellular matrix assembly binding with laminin and also with collagen IV and fibronectin.

(2) Role in homeostasis and wound healing by interacting with fibrinogen [253]. NID1 is a prosurvival and promigratory factor for adult Schwann cells. Schwann cells provide a favourable microenvironment for the successful regeneration of the injured peripheral nerve. A study showed that the mRNA messages of NID1 are upregulated in the sciatic nerve after sciatic nerve transection [257].

(3) Enhancement of cell attachment by association with some receptors as integrins [253].

(4) Enhancement of chemotaxis and phagocytosis, most likely through specific integrin receptors [258, 259].

To understand the physiological role of the nidogens in basement membranes, different mouse models were studied. The lack of nidogen-1 in mice does not affect basement membrane formation. Instead, nidogen-1 -/- mice develop seizures and other neurological defects later in life [260]. This phenotype could be explained by the existence of the structurally related nidogen-2/entactin-2 [261, 262] and a possible compensatory effect of nidogen-2 in basement membrane assembly of nidogen-1 deficient mice. Although immunostaining for nidogen-2 is stronger in distinct basement membranes, it does not appear to be transcriptionally upregulated in nidogen-1 -/- mice. Nidogen-2 null mutant mice also appear normal. The production of nidogen-1/-2 double null-mutant mice is currently underway (Smyth, personal communication). This will help to explain the importance of nidogen-1 and -2 and their contribution to basement membrane stability.

7.3 Nidogen 1 and cancer

To date, the association of *NID1* with cancer has been rarely reported.

Lee et al. demostrated that the ectodomain of tumour endothelial marker seven (TEM7) interacts with *NID1* with relatively high affinity. TEM7 is a transmembrane protein highly expressed in tumour endothelia, cerebellar Purkinje cells, and the CA1-CA3 layers of the hypothalamus. They demonstrated that cells transfected with *TEM7* showed extensive spreading when plated on NID1, suggesting that further nidogen receptors may exist [263].

The *NID1* gene has been described as a biologically plausible candidate locus for nevogenesis and melanoma development, since NID1 is a major component of the basement membrane in the skin and it plays an active role in BM membrane assembly, i.e., it interacts with many other BM molecules, promoting the components that integrate the elements for BM assembly [264].

Conversely, in human gastrointestinal cancer *NID1* and *NID2* gene promoters are aberrantly methylated, which suggests that loss of the expression of nidogens has a potential pathogenetic role in colon and stomach tumourigenesis [265]. Ulazzi et al. reported that CpG islands of both *NID1* and *NID2* genes are aberrantly methylated in human cancer samples and cancer cell lines. Futhermore, demethylation of *NID1* and

NID2 promoters restored gene transcription, proving that methylation was responsible for silencing nidogen genes [265].

Recently, Zhang et al. described NID1 as a potential biomarker in blood for ovarian cancer. Plasma proteomic studies using LC-MS/MS (liquid chromatography tandem mass spectrometry) have reported significantly higher levels of NID1 in plasma samples from patients with ovarian cancer compared with healthy controls [266].

OBJECTIVES

Background

Endometrial cancer is the most frequent malignancy of the female genital tract in Western countries. The early appearance of symptoms, most commonly abnormal vaginal bleeding and high abdominal pain in post menopausal women [100], contributes to the earlier detection of this malignancy and results in better survival rates. However, in 20% of cases the diagnosis is delayed and patients present with myometrial infiltration and/or lymph node affectation. Myometrial invasion, an initial event that signals tumour invasion is one of the most valuable prognostic factors and it determines increased recurrence rates after the first surgical treatment and decreased 5-year survival. Consequently, unravelling the initial steps associated with myometrial infiltration is fundamental to identify new therapeutic agents for the prevention of cancer dissemination.

To this end, the major scientific aim of our group during the past few years has been the identification of the molecular mechanisms involved in endometrial cancer invasion and dissemination. The gene expression analysis that compared endometrial tumours with endometrial normal and atrophic tissue lead to the identification of the ETV5 transcription factor as an important agent of myometrial invasion by endometrial tumour cells. ETV5 is specifically upregulated in invasive endometrial tumours and is able to [147] activate the metalloproteinase MMP2, thus promoting cell migration and invasion *in vitro* and *in vivo* [148]. In addition, we demonstrated the role of ETV5 on the induction of EMT through the upregulation of ZEB1 expression and E-cadherin repression, which result in the acquisition of migratory and invasive capabilities. We have also identified the lipoma-preferred partner (LPP) as a regulatory partner of ETV5; LPP acts as a sensor for extracellular signals that promote tumour invasion [137].

Summary of the objectives

The main objective of this thesis is to identify new molecules involved in the myometrial invasion of endometrial cancer. In particular, we have focused on the identification of new ETV5 downstream targets that mediate the role of ETV5 in the endometrial tumour cells' acquisition of migratory and invasive properties.

General objectives

1) Downstream pathways regulated by ETV5 in endometrial cancer cells.

To further characterise the initial steps of myometrial invasion regulated by the ETV5 transcription factor, we analysed by gene expression microarray technology those genes whose expression was altered in Hec1A endometrial cancer cells with stable overexpression of a fusion GFP-ETV5 protein, compared with Hec1A control cells. In the first study presented in this thesis we analysed the genes and downstream pathways regulated by ETV5.

Specific objectives:

a) Analysis of genes and pathways regulated by ETV5 using the Ingenuity Pathway Analysis software.
b) Selection of putative ETV5 target genes by statistical analysis, literature mining and promoter analysis.
c) ChIP assays on ETV5 putative gene targets selected in section b.
d) Validation of altered mRNA and protein expression of genes selected in section c due to ETV5 modulation in endometrial cancer cells.
e) Analysis of ETV5 transcriptional regulation on selected genes by gene reporter assay.

2) *In vitro* effects of knowcking down NID1 and NUPR1 in Hec1A endometrial cancer cells overexpressing ETV5.

In particular, we have identified *NID*1 and *NUPR1* as direct transcriptional targets of ETV5. NID1 is a ubiquitous protein component of the BM and a partner of laminin. NUPR1 is a small protein involved in different types of cancer that can act as oncogene and also as tumour suppressor depending on the type of cancer. NUPR1 is a HMG protein and its principal role in the cell is to bind to the chromatin promoters to modify the accessibility of chromatin in gene regulation.
Our objective was to examine the role of NID1 and NUPR1 as mediators of ETV5 functions in Hec1A endometrial cancer cells *in vitro*.

Specific objectives:

a) Down-regulation of *NID1* and *NUPR1* by shRNA in Hec1A EC cells with ETV5 overeexpression (HGE).
b) Functional analysis of HGE cells with inhibition of NID1 and NUPR1 using proliferation, migration and invasion *in vitro* assays
c) Functional analysis of HGE cells with inhibition of NID1 using an *in vitro* adhesion assay.
d) Functional analysis of HGE cells with inhibition of NUPR1 using an apoptosis *in vitro* assay.

3) *In vivo* effects of knocking down NID1 and NUPR1 in Hec1A endometrial cancer cells overexpressing ETV5.

As in objective number 2, we wanted to examine the role of NUPR1 as a mediator of ETV5 functions in Hec1A endometrial cancer cells *in vivo* using an orthotopic mouse model. Our group has recently established an orthotopic mouse model of endometrial cancer which features cancer cells growing in their natural location and replicates human disease with high accuracy [267]. In this model, endometrial cancer cells are injected into the mouse's uterine cavity, where the tumour grows and invades adjacent organs following myometrial invasion.

Specific objectives:

a) Analyze HGE cells with inhibition of NID1 and NUPR1 in tumour progression *in vivo* assays.
b) Analyze HGE cells with inhibition of NID1 and NUPR1 in tumour dissemination and metastases *in vivo* assays.

4) Role of NID1 and NUPR1 in human endometrial cancer.

In previous studies, ETV5 was upregulated in human endometrial tumour samples compared with control tissue, in particular in the invasion front of endometrial tumours [137]. We wanted to examine the role of both NID1 and NUPR1 genes in human endometrial tumour samples and their association with clinical and pathological characteristics.

Specific objectives:

a) To measure *NID1*, *NUPR1* and *ETV5* mRNA expression levels in human endometrial tumour samples.
b) To measure *NID1*, *NUPR1* and *ETV5* expression in superficial and deep tumour samples at mRNA level.
c) To measure NID1, NUPR1 and ETV5 protein expression using a multiple stage TMA.
d) To measure NID1, NUPR1 and ETV5 protein expression in superficial and deep tumour samples TMA.

MATERIALS AND METHODS

1 Collection of human samples

Tumour samples

In this thesis, samples from a group of patients who underwent surgery for endometrial carcinoma in the Departments of Gynaecological Oncology at the University Hospital Vall d'Hebron and the University Hospital del Mar in Barcelona have been analyzed. Patients' age ranged from 50 to 80 years. None of the patients had received radiation, hormonal therapy or chemotherapy prior to surgery. The protocol had been previously approved by the Institutional Review Boards (code 120/2005), and informed consent was obtained from all participants. After surgery, each tissue sample was immediately examined and dissected by a pathologist and subsequently stored paraffin-embedded at -80 °C until analysis.

For RNA expression analyses, 32 samples of EEC at different FIGO stages and grades were used to analyze the correlation of *NID1*, *NUPR1* and ETV5 in human tumour samples (Table 7), while 13 paired samples of superficial and deep tumour were used to analyze *NID1* and *NUPR1* expression in the invasion front of the endometrial tumour (Table 8).

A tissue microarray that included 82 tissue samples from different tumour types and grades and controls was used to analyze the protein expression levels of NID1, NUPR1 and ETV5 in human tumour samples (Table 9). An additional TMA including 116 tumour paired samples from superficial and deep invasive tumour was used to analyze NID1 and NUPR1 and ETV5 protein expression at the invasion front of endometrial tumours (Table 10).

Table 7. Histopathological characteristics of the tumour endometrial samples used to analyze the expression levels of NID1 and NUPR1 by RTqPCR.

Sample	Histological type	FIGO stage	Grade
1	EEC	Ia	G2 (Moderately differentiated)
2	EEC	Ia	G2
3	EEC	Ia	G1
4	EEC	Ia	G1 (Well differentiated)
5	EEC	Ia	G1 (Well differentiated)
6	EEC	Ib	G2 (Moderately differentiated)
7	EEC	Ib	G2 (Moderately differentiated)
8	EEC	Ib	G2 (Moderately differentiated)
9	EEC	Ib	G2 (Moderately differentiated)
10	EEC	Ib	G3 (No differentiated)
11	EEC	Ic	G2 (Moderately differentiated)
12	EEC	Ic	G2
13	EEC	Ic	G2 (Moderately differentiated)
14	EEC	Ic	G3 (No differentiated)
15	EEC	Ic	G2 (Moderately differentiated)
16	EEC	Ic	G2 (Moderately differentiated)
17	EEC	Ic	G3
18	EEC	Ic	G3
19	EEC	Ic	G2 (Moderately differentiated)
20	EEC	Ic	
21	EEC	IIa	G2
22	EEC	IIa	G2
23	EEC	IIb	G3 (No differentiated)
24	EEC	IIb	G2 (Moderately differentiated)
25	EEC	IIb	G2 (Moderately differentiated)
26	EEC	IIb	G2 (Moderately differentiated)
27	EEC	IIb	G2
28	EEC	IIIa	G2 (Moderately differentiated)
29	EEC	IIIa	G3 (No differentiated)
30	EEC	IIIa	G3 (No differentiated)
31	EEC	IIIc	G3
32	EEC	IVb	G2 (Moderately differentiated)

Table 8. Histopathological characteristics of tumour endometrial samples used to analyse the expression levels of NID1 and NUPR1 by RTqPCR in superficial and deep tumour sections.

Sample	Histological type	FIGO stage	Grade
1	EEC	Ic	G1
2	EEC	Ic	G3
3	NEEC	Ic	G3
4	EEC	Ic	G2
5	EEC	Ic	G3
6	NEEC+EEC	Ic	G3
7	EEC	Ic	G3
8	EEC	Ic	G2
9	EEC	Ic	G3
10	Undifferentiated Ca	Ic	G3
11	EEC	Ic	G2
12	Carcinosarcoma	Ic	G3
13	EEC	Ic	G2

Table 9. Histopathological characteristics of the tumour endometrial samples included in the TMAs and used to analyze by IHC the expression levels of NID1 and NUPR1.

Sample	Histological Type	FIGO stage	Grade	Sample	Histological Type	FIGO stage	Grade
1	EEC	Ib	G2	**41**	Clear Cell Ca	IVb	G3
2	Clear Cell Ca	IIb	G3	**42**	EEC	IIb	G3
3	EEC	IIa	G2	**43**	Carcinosarcoma het.	IIIc	G3
4	EEC	Ib	G2	**44**	EEC	IIb	G2
5	EEC	Ia	G1	**45**	Serous Ca	IIIc	G3
6	EEC	Ib	G2	**46**	EEC	IIb	G3
7	Clear Cell Ca	IIIc	G3	**47**	EEC	IIb	G2
8	EEC	IIb	G2	**48**	EEC	IIb	G3
9	EEC	Ib	G2	**49**	Serous Ca	IIIc	G3
10	EEC	Ib	G3	**50**	EEC	IIIa	G2
11	EEC	Ia	G1	**51**	EEC	Ib	G1
12	EEC	Ib	G2	**52**	EEC	Ib	G2
13	EEC	Ib	G2	**53**	Serous Ca	IIIa	G3
14	EEC	Ia	G3	**54**	EEC	Ib	G2
15	EEC	IIb	G3	**55**	EEC	Ib	G3
16	Serous Ca	IIIc	G3	**56**	EEC	IIb	G2
17	Serous Ca	IIa	G3	**57**	EEC	Ib	G3
18	EEC	Ib	G2	**58**	Atrophic E		
19	EEC	IIb	G2	**59**	Secretor E		
20	EEC	Ib	G2	**60**	EEC	Ic	G2
21	EEC	Ic	G2	**61**	Serous Ca	Ib	G3
22	EEC	Ib	G3	**62**	Atrophic E		
23	EEC	Ia	G2	**63**	Proliferative E		
24	EEC	Ic	G2	**64**	EEC	Ia	G1
25	Adenosquamous Ca.	IIIa	G2	**65**	Villoglandular Ca	Ib1	G2
26	EEC	IIb	G3	**66**	EEC	Ib	G3
27	EEC	Ib	G1	**67**	Atrophic E		
28	EEC	IIb	G2	**68**	Proliferative E		
29	EEC	Ib	G2	**69**	Carcinosarcoma	Ib	G3
30	Carcinosarcoma	Ib	G3	**70**	EEC	Ib	G2
31	EEC	Ia	G1	**71**	EEC	Ic	G2
32	EEC	Ic	G3	**72**	Complex Hyperplasia		
33	EEC	Ib	G1	**73**	Proliferative E		
34	EEC	Ic	G2	**74**	EEC	Ib	G2
35	EEC	IIa	G1	**76**	EEC	IIb	G2
36	EEC	Ib	G1	**77**	Simple Hyperplasia		
37	EEC	IIb	G2	**78**	Atrophic E		
38	EEC	Ic	G3	**79**	EEC	Ia	G2
39	Clear Cell Ca	III	G3	**80**	EEC	IIa	G1
40	EEC	Ib	G2	**81**	Mucinous Ca	Ib2	G2
				82	Simple Hyperplasia		

Table 10. . Histopathological characteristics of tumour endometrial samples included in the TMAs and used to analyse by IHC the expression levels of NID1 and NUPR1 in superficial and deep tumour sections.

Sample	Histological Type	FIGO stage	Grade	Sample	Histological Type	FIGO stage	Grade	Sample	Histological Type	FIGO stage	Grade
1	EEC	Ia	G1	39	EEC	Ia	G1	78	EEC	Ia	G1
2	EEC	Ia	G1	40	EEC	Ib	G1	79	EEC	Ia	G1
3	EEC	Ia	G1	41	EEC	II	G3	80	EEC	Ib	G2
4	EEC	Ia	G2	42	EEC	Ia	G1	81	EEC	Ib	G2
5	EEC	Ia	G1	43	EEC	II	G3	82	EEC	Ia	G1
6	EEC	Ia	G1	44	EEC	Ia	G2	83	EEC	Ia	G1
7	EEC	III	G3	45	EEC	Ib	G2	84	EEC	II	G3
8	EEC	Ia	G2	46	EEC	Ib	G1	85	EEC	Ia	G1
9	EEC	Ib	G1	47	EEC	Ib	G1	86	EEC	Ib	G2
10	EEC	Ib	G3	48	EEC	Ia	G1	87	EEC	Ia	G2
11	EEC	Ia	G1	49	EEC	Ib	G3	88	EEC	Ib	G2
12	EEC	Ia	G1	50	EEC	Ia	G1	89	EEC	Ib	G1
13	EEC	Ib	G2	51	EEC	Ia	G1	90	EEC	Ib	G2
14	EEC	Ib	G1	52	EEC	Ia	G1	91	EEC	II	G1
15	EEC	Ib	G1	53	EEC	Ib	G2	92	EEC	Ib	G1
16	EEC	Ia	G1	54	EEC	II	G1	93	EEC	Ia	G1
17	EEC	II	G1	55	EEC	Ia	G1	94	EEC	Ia	G1
18	EEC	Ia	G1	56	EEC	Ib	G1	95	EEC	Ia	G2
19	EEC	Ib	G1	57	EEC	Ib	G1	96	EEC	Ia	G1
20	EEC	Ib	G3	58	EEC	Ia	G1	97	EEC	III	G3
21	EEC	II	G1	59	EEC	Ia	G2	98	EEC	II	G2
22	EEC	III	G3	60	EEC	Ia	G1	99	EEC	II	G3
23	EEC	Ia	G1	61	EEC	Ib	G1	100	EEC	Ia	G1
24	EEC	Ib	G2	62	EEC	Ib	G1	101	EEC	Ia	G1
25	EEC	Ia	G1	63	EEC	Ib	G1	102	EEC	Ib	G1
26	EEC	Ia	G1	64	EEC	Ib	G1	103	EEC	Ia	G1
27	EEC	Ib	G1	65	EEC	Ib	G3	104	EEC	Ib	G3
28	EEC	Ia	G1	66	EEC	Ia	G2	105	EEC	Ib	G1
29	EEC	Ia	G1	67	EEC	Ia	G1	106	EEC	Ib	G1
30	EEC	Ia	G1	68	EEC	Ib	G1	107	EEC	Ib	G1
31	EEC	Ia	G2	69	EEC	Ia	G1	108	EEC	Ia	G1
32	EEC	Ib	G1	70	EEC	Ib	G2	109	EEC	II	G1
33	EEC	II	G2	71	EEC	Ia	G1	110	EEC	Ib	G1
34	EEC	Ia	G2	72	EEC	Ib	G1	111	EEC	II	G1
35	EEC	Ib	G1	73	EEC	Ib	G2	112	EEC	II	G2
36	EEC	Ia	G1	74	EEC	III	G2	113	EEC	Ia	G1
37	EEC	Ia	G1	75	EEC	Ia	G1	114	EEC	Ia	G1
38	EEC	Ia	G1	76	EEC	II	G3	115	EEC	Ia	G1
				77	EEC	II	G1	116	EEC	Ib	G1

Control samples

We collected non-affected endometrial epithelia (AE: atrophic epithelia) from the same patients to use as control tissue in the mRNA expression analysis.

2 Human cell lines

2.1 Endometrial cancer cell lines

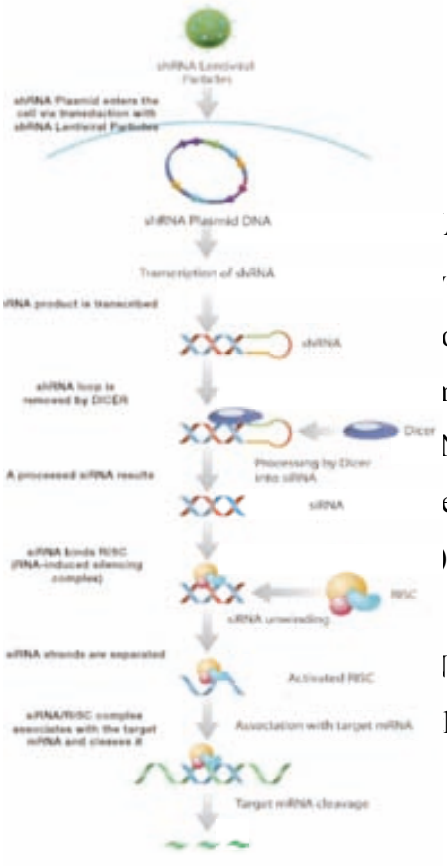
Human cell lines are used in most research laboratories to study tumour characteristics including cell biology, genetics, and the chemosensitivity profile of diseases. A large number of endometrial cancer cell lines have been established, and for this study we used mainly the human endometrial carcinoma cell line Hec1A.

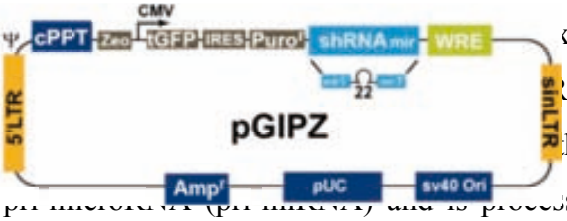
Hec1A cell line was cultured in McCoy's 5A with L-Glutamine HyClone medium (Thermo Scientific), supplemented with 10% foetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad), Fungizone (3.5 mL in 500ml of medium, Gibco) and Penicillin-Streptomycin (1:100;Gibco) at 37ºC in a humidified atmosphere of 5% CO_2.
The AN3CA cell line was cultured in DMEM F:12 (Thermo Scientific), supplemented with 10% foetal bovine serum (FBS; Gibco, Invitrogen, Carlsbad), Fungizone (3.5 mL in 500ml of medium, Gibco) and Penicillin-Streptomycin (1:100;Gibco) at 37ºC in a humidified atmosphere of 5% CO_2.

2.2 Constructs and generation of stable cell line

In order to overexpress the ETV5 transcription factor in Hec1A endometrial cells, ETV5 was cloned in the pEGFP-C2 vector (BD Biosciences). Next, Hec1A (H) were transfected with either the pEGF-C2 vector alone (HG) or the hERM/ETV5 containing pEGF-C2 vector (HGE). Hec1A cells stably expressing green fluorescent protein (GFP) or GFP-ERM-ETV5 were established by selection with 500 μg/mL geneticin G418 (Life Technologies).

In order to suppress *NID1* and *NUPR1* expression in HGE cells, short hairpin RNAs against both genes were used.





As ( ) ...pression of selected ...ecto... ...RNA is transcribed in olym... ...he promoter choice. ...nics pri-microRNA (pri-miRNA) and is processed by Drosha. The ...NA is exported from the nucleus by Exportin 5. This product is then ...er and loaded into the RNA-induced silencing complex (RISC). The ... strand is degraded. The antisense (guide) strand directs RISC to ... complementary sequence. In case of perfect complementarity, RISC ...A. In case of imperfect complementarity, RISC represses mRNA ...h cases, the shRNA leads to targeted gene silencing (Figure 19A).

B)

Figure 19. A) shRNA inhibits the specific gene. B) pGIPZ vector used to inhibit NID1 and NUPR1 genes in EC cell lines.

The human pGIPZ lentiviral shRNAmir target gene set (Thermo Scientific) was used to knock-down *NID1* (V3LHS_300641) and *NUPR1* (V3LHS_307648) expression in

HGE cells. The control short hairpin consisted in a short hairpin RNA that silences a gene which is not transcriptionally active.

To generate stable cell lines with downregulation of *NID1* and *NUPR1*, we used the lentiviral vectors carrying a CMV-driven Lac Z gene packaged with attenuated HIV-derived constructs and pseudotyped with VSV-G envelope (provided by J. Seoane from the Gene Expression and Cancer Department in the Vall d'Hebron Research Institute), prepared by transient transfection of 293T cells [268], together with pGIPZ against NID1, NUPR1 or control. The transfection was carried out using Lipofectamine 2000 (Invitrogen), following manufacturer's instructions.

One day after transfection, the medium of 293T cells was replaced with McCoy's medium. Lentiviral supernatants were harvested 48h after transfection and were filtered through 0.45 μm. Cleaned lentiviral medium was used to infect cells in the presence of 8 μg/ml polybrene. Efficiency of infection was assessed by RTqPCR and WB. Transfected cells were next selected with Puromicin (InvivoGen) at 1 μL/mL.

3 Gene expression analysis

3.1 RNA extraction

Human samples

Total RNA was collected and purified using the RNeasy kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. This technology combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows RNA longer than 200 bases to bind to the silica-gel membrane. In the RNA purification protocol, biological samples are first lysed and homogenized in the presence of a highly denaturing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to a mini column where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30 μl or more of RNase-free water.

Total isolated RNA was measured by Bioanalyzer Assay (Agilent, Santa Clara, CA). This microfluidics-based platform is used for sizing, quantification and quality control

of DNA, RNA, proteins and cells. It allows a quick and sensitive investigation of nucleic acid samples. In addition to measuring total RNA quantity, this technique can also determine RNA integrity (RNA Integrity Number, RIN).

Cell lines

Before RNA extraction, cultured cells were washed with PBS 1x and scrapped. Cell suspensions were centrifuged and washed again with PBS 1x. Pellets obtained after centrifugation were used to extract RNA and DNA simultaneously. Total RNA was collected and purified using the RNeasy kit (Qiagen), following manufacturer's instructions.

The quantity of isolated RNA was measured by Nanodrop (Thermo Scientific). This micro-volume spectrophotomer allows the determination of the absorbance at 260 nm (RNA) and 280 nm (protein) in small volume samples. Sample purity value is given as a ratio of 260/280 nm below 2.

3.2 Retrotranscription (RT)

Retrotranscription (RT) is a widely used process by which RNA is converted to DNA. In this process an inverse retrotranscriptase (DNA polymerase) is used to obtain a complementary cDNA from mRNA when it is mixed with other reagents such as dNTPs and primers. cDNA is more stable than mRNA because RNA is very easily degraded by omnipresent RNases. Retrotranscription is needed prior to the determination of gene expression levels inside the cells using PCR or RTqPCR.

In our gene expression experiments, two micrograms of total purified RNA were subjected to a reverse transcriptase reaction using 1 µl of Random Primers (50 µM, Invitrogen), 1 µl of dNTPs mixture (10 mM, Promega) and sterile distilled water to 13 µl for each reaction. Retrotranscription was performed using a thermocycler under the following conditions: 5 min at 65 ºC, followed by an incubation of 1 minute on ice; addition of 4 µl of 5X First Strand Buffer, 1 µl of DTT (0.1 M, Invitrogen) and 1 µl of SuperScript III (Invitrogen), followed by an incubation for 5 min at 25 ºC, 60 min at 50ºC and 10 min at 70 ºC, with a final step at 4 ºC.

3.3 Polymerase chain reaction (PCR)

The PCR assay was developed by Kary Mullis in 1983. It amplifies a single or a few copies of a DNA segment across several orders of magnitude, generating thousands to millions of copies of a specific DNA sequence. Almost every PCR application employs a heat-stable DNA polymerase, such as Taq polymerase, which assembles a new DNA strand by using single stranded DNA as a template and DNA oligonucleotides under the following thermal cycling conditions: 95 ºC during 5 minutes for a hot start, followed by 30-40 cycles at 94 ºC during 30 seconds to denature the DNA, then 30 seconds at the specific primer temperature and to finish the cycle, 30 seconds at 74 ºC to extend the DNA, and after the 30-40 cycles the last step consists in 72 ºC for 10 minutes. The annealing temperature depends on the base composition of the two primers and is calculated by applying the formula (C+G) *4 + (A+T) *2 to each primer. Next, the lowest temperature is chosen and 2 ºC are subtracted to reach the final temperature.

3.4 Real Time quantitative PCR (RTqPCR)

Real-time quantitative PCR (RTqPCR) is a PCR technique used to measure the quantity of a PCR product in real-time. It quantitatively measures starting amounts of DNA, cDNA, or RNA. RTqPCR is mostly used to determine whether a DNA sequence is present and the number of copies found in the sample. It is highly precise thanks to the use of fluorophore-containing DNA probes such as TaqMan. We used RTqPCR to quantify and verify gene expression data in different types of samples: 1) in endometrial superficial and deep tumour samples and endometrial control samples; 2) and in endometrial cancer cell lines. cDNA corresponding to approximately 2 µg of starting RNA was used in three replicates for quantitative PCR (Taqman, Applied Biosystems, Foster City, CA, USA). We used TaqMan probes to determine levels of expression of the genes of interest.

RTqPCR of human samples

For the analysis of endometrial tumour samples and controls and of superficial and deep tumour, we used the ETV5-Hs00231790_m1, NID1-Hs00159600_m1 and

NUPR1-Hs00202610_m1 probes. The GAPDH-Hs9999905_m1 probe was used for normalization (Applied Biosystems).

RTqPCR of cell lines

For the analysis of endometrial cancer cell lines, we used the ETV5-Hs00231790_m1, NID1-Hs00159600_m1 and NUPR1-Hs00202610_m1 probes, and the 18s ribosomal RNA C6_4308329 probe for normalization (Applied Biosystems).

4 Protein expression analysis

4.1 Protein extraction

Total protein extraction from cells

Before protein extraction, cultured cells were scrapped with 1 mL of PBS 1x. Pellets obtained after centrifugation during 2 minutes were used to extract total protein. Whole cell extracts were prepared using RIPA Buffer (Tris 20 mM pH8.8, NaCl 150 mM, EDTA 5 mM, Triton X-100 1%, protease inhibitors) and incubated in ice for 1 hour. After 15 minutes of centrifugation, supernatants containing proteins were quantified by BioRad DC™ Protein Assay (Reagent A, Reagent B and Reagent S, BioRad) and then boiled with Laemmli Buffer (100 mM Tris−HCl pH6.8, 4% SDS and 20% glycerol) during 5 minutes at 95 ºC. After centrifugation, the protein fractions were stored at -20 ºC.

Nuclear-Cytoplasmic protein extraction from cells

Nuclear and cytoplasmic protein was extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Cat No. 78835, Thermo Scientific, USA), following manufacturer's instructions. Adherent cells were harvested with trypsin and centrifuged at 500 g during 5 minutes. Cells were washed with PBS1x and centrifuged at 500 g for 3 minutes. Supernatants were discarded and cold CERI reagent added. After incubation on ice, CER II reagent was added. Next, samples were centrifuged at maximum speed during 5 minutes and the supernatant corresponding to the cytoplasmic fraction was stored at -80 ºC. The pellet was then resuspended with NER reagent and placed on ice with a continued vortexing for 15 seconds every 10 minutes

for a total of 40 minutes. After a maximum speed centrifugation, the pellet corresponding to the nuclear fraction was stored at -80 ºC.

TCA Protein Precipitation

Adherent cells were cultured in a p100 dish in 6 mL of serum-free medium during 48 hours. The medium was collected in a sterile tube and 6 mL of 20% trichloroacetic acid (TCA) was added drop by drop with agitation. The sample was incubated 10 minutes on ice and centrifuged at 10,000 g during 20 minutes. The pellet was resuspended with 6 mL of 10% TCA. Next, the sample was centrifuged at 10,000 g during 20 minutes. Finally, the pellet was washed with 3 mL of diethyl ether and centrifuged for 10 minutes at 10,000g. The pellet was finally resuspended with RIPA Buffer 1% SDS.

4.2 Western Blot

Western Blot (WB) is a technique used to detect specific proteins in homogenate tissue samples or cellular extracts. It uses gel electrophoresis to separate denatured proteins by length and electric charge. The proteins are then transferred to a membrane of nitrocellulose or PVDF, where they are detected using antibodies specific to the target protein.

Samples were run on 10% (for NID1 and ETV5) and 15% (for NUPR1) SDS−polyacrylamide gel and transferred to a PVDF membrane (Bio−Rad Laboratories, Hercules, CA, USA). Membranes were blocked in a 5% milk solution (TBS−0.1% Tween) for 1h at room temperature and incubated with the primary antibody in 5% milk solution overnight at 4 °C. The membranes were washed three times for 10 min in TBS−0.1% Tween at room temperature and incubated for 1 hour with the corresponding HRP conjugated secondary antibody. Proteins were detected by the enhanced chemiluminiscence system (Amersham Pharmacia Biotech), following manufacturer's instructions.

For the endometrial cancer cell line protein extracts, the primary antibodies used for Western blot were as follows: anti−ERM/ETV5 (sc−22807, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti−NID1 (anti-hNidogen1, MAB2570,

R&D Systems, Minneapolis, MN USA), anti-NUPR1 anti-rat monoclonal antibody provided by Dr. Iovanna from the Cellular Stress Laboratory (INSERM, Marseilles), anti-E-cadherin (610181) and anti N-cadherin (610920, BD Transduction Laboratories Biosciences Pharmigen).

4.3 Immunohistochemistry

Immunohistochemistry (IHC) is a technique used to detect antigens (proteins) in cells of a tissue section using specific antibodies. To visualize an antibody−antigen interaction, the secondary antibody is conjugated to an enzyme such as peroxidase that will catalyze a colour producing reaction. In our studies, we performed IHC staining in tumour tissue slides and in endometrial tumour tissue microarrays.

IHC in tissue slides and in tissue microarrays

Tissue microarrays (also TMAs) consist of paraffin blocks in which up to 1000 separate tissue cores are assembled in array fashion to allow multiplex histological analysis.

Two tissue microarrays were constructed at the Pathology Department of the Vall d'Hebron University Hospital. Representative areas from 82 paraffin-embedded tissues from different types and grades of carcinomas and controls (Table 9) were carefully selected and marked on individual paraffin blocks. Two tissue cores of 1 mm in diameter were obtained from each paraffin block and were precisely arrayed in a new paraffin block. 5 μm sections were obtained from all tissue microarray paraffin blocks. NID1 and ETV5 were detected by the indirect immunoperoxidase assay with citrate buffer pH 9 for antigen retrieval. Sections were incubated with primary antibodies for 2 h at room temperature using 1:25 and 1:100 dilutions, respectively. Next, sections were incubated with peroxidase conjugated goat anti-rabbit and anti-mouse immunoglobulin (EnVision Dual System, DAKO). Endogenous peroxidase activity was quenched with 3% H_2O_2. Sections were washed, and reactions were developed with diaminobenzidine, followed by counterstaining with hematoxylin. The antibodies used for IHC staining were anti−ERM/ETV5 (sc−22807, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti−NID1 (anti-hNidogen1, MAB2570, R&D Systems, Minneapolis, MN USA), and an anti-NUPR1 anti-rat monoclonal

antibody provided by Dr. Iovanna from the Cellular Stress Laboratory (INSERM, Marseilles). NUPR1 was detected in collaboration with the Cellular Stress Laboratory directed by Dr. Iovanna (INSERM, Marseilles).

An additional TMA was constructed at the Pathology Department of the Hospital del Mar (Barcelona). Representative areas from 116 paraffin-embedded tissues from superficial and invasion front paired samples (Table 10) were carefully selected and marked on individual paraffin blocks. 5 μm sections were obtained from all tissue microarray paraffin blocks. NID1, ETV5 and NUPR1 were detected as described before.

Semiquantitative evaluations of both proteins were performed by an experienced pathologist, who determined percentage of positive cells.

5 Signalling pathway analysis

Functional pathway and network analyses were performed by means of the use of Ingenuity Pathway Analysis (version 9.0, Ingenuity® Systems, Mountain View, CA). IPA can integrate data from a variety of experimental platforms and provides insight into the molecular and chemical interactions, cellular phenotypes and disease processes of the system. IPA identified those canonical pathways, biological processes and gene interaction networks that were most significant to the genes selected from the microarray expression analysis (differentially expressed genes with log FC <-1.3; >1.3 and an adjusted p–value <0.01).

6 Chromatin Immunoprecipitation Assay (ChIP)

Chromatin Immunoprecipitation (ChIP) is a type of experimental immunoprecipitation technique used to investigate the interaction in the cell between proteins and DNA. It aims to determine whether specific proteins are associated with specific genomic regions, for instance, transcription factors with promoters or other DNA binding sites [269].

Briefly, protein and associated chromatin in a cell lysate are temporarily bonded, the DNA-protein complexes (chromatin-protein) are then sheared and DNA fragments associated with the proteins of interest are selectively immunoprecipitated, the associated DNA fragments are purified and their sequence is determined. These DNA sequences are supposed to be associated with the protein of interest *in vivo*.

Chromatin immunoprecipitation (ChIP) on Hec-1A GFP-ERM/ETV5 cells was performed using rAb ERM/ETV5 and the Magna ChIP kit (Millipore, Billerica, MA), according to manufacturer's instructions. Total input and DNA immunoprecipitated with rAb α-acetyl-histone H3 were used as positive controls, and DNA immunoprecipitated with α-normal rabbit IgGs and non-antibody were used as negative controls. For PCR analysis, 1 µl of input DNA extraction and 5 µl of immunoprecipitated DNA were used for 35 cycles of amplification. One or two sets of primers covering the *ANGPT-2, PLOD-2, NUPR1, EFNA5, NPR3, NID1, ICAM1, LAMP3* and *CD44* putative ETS binding sites were designed to amplify the promoter regions of ETV5 putative target genes. Melting temperatures and specific PCR conditions are described in the table below.

Table 11. List of primers and PCR conditions used for ChIP analysis of promoter regions. GAPDH was used as a loading control.

Primer gene	Forward	Reverse	5% DMSO	Annealing temp.
ANGPT-2	5'GAAGTCCTGACCTATTTGTAG3'	5'TCTCCCCAGATCCTACAGTG3'	no	58 ºC
PLOD2a	5'GAA AGA GTC TAA GGC TCT CTT G3'	5'CAC TTA GCT TTG GGA GTG GGT T3'	yes	56ºC
PLOD2b	5'AAC CCA CTC CCA AAG CTA AGT G3'	5'GCA GCT GAG GCT TCA CCG TGC3'	yes	56ºC
NUPR1a	5'-CATTTGATCCTCTCCCCAAC-3'	5'-GATTATAGACATCTGCCACC-3'	no	54ºC
NUPR1b	5'-AAGTGTGCTGATATCCCTTC-3'	5'-CTTCTCCTAACGCTTTGTCT-3'	no	51ºC
EFNA5a	5' GAG TTG GAG GGA TCC ATT TG3'	5' GAT AGA GGG CTT CGC GCT TG3'	no	50ºC
EFNA5b	5'ACA CAG CTT GGC ACC TCT TC3'	5'CAT CTC CAC GTG CAA CAT CAC3'	no	52ºC
NPR3a	5'ATT CCA GCG CAA ACC TGC GTG3'	5'GGA GTC TCT CAT TAA CAT TCT3'	yes	58ºC
NPR3b	5'CGC TGC CAC GCT ATT TAA AC3'	5'CAA GAA AGA GCT TGC CCT C3'	no	60ºC
NID1a	5'-GTTTCTTCTCCTCTTCAATGC-3'	5'-CCAAGTCATCAAAGACATTAG-3'	yes	60ºC
NID1b	5'-TTTCCACGTCGCCGGCTCTC-3'	5'-CATGTTCCCGAACTGCGGTC-3'	yes	60ºC
LAMP3a	5'CAG CTG AAG AAT CCA AGG CTC3'	5'TTT TCT AAC AGC TCG GGT ACC3'	no	62ºC
LAMP3b	5'CAG CCT GGC CAA TAT GGT GAC3'	5'GTT CTG CAG CGT GCG GCG AAG3'	yes	62ºC

7 Promoter Reporter Assay

A reporter gene is a gene attached to a regulatory sequence of another gene of interest. The reporter gene is placed under the control of the target promoter and the reporter gene product's activity is quantitatively measured. In this case the reporter is firefly luciferase, which is directly attached to the promoter of interest (NID1 or NUPR1 promoter).

The human NID1 and NUPR1 promoters (promoter region comprising nucleotides from -375 to 3 and from -444 to -30, respectively) were cloned in the pGL4.15 luciferase vector (pGL4.15-NID1 and pGL4.15-NUPR1). Cells were split into 24-well plates before transfection and were transfected with a mixture of 370 ng reporter luciferase vector, 30 ng renilla luciferase vector and Lipofectamine 2000 (Invitrogen) for 5 h with serum-free medium. The transfection mixture was replaced with complete medium. After 36 h, cells were lysed and luciferase activity determined using the Dual Luciferase Assay Kit (Promega). Data are presented as relative fold activation between cells transfected with either pGL4.15-NID1 and pGL4.15-NUPR1 and the pGL4.15 empty vector. At least three independent transfection experiments were performed.

8 Cell proliferation assay

Cell proliferation can be detected by a variety of methods. Quantifying cell viability in a proliferation assay is crucial for understanding cancer biology, compound toxicity and cellular response to cytokines.

In our proliferation assays, we used the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) kit, a colorimetric method for determining the number of viable cells. It uses the MTS tetrazolium compound, which is bioreduced by cells into a formazan product, which can then be detected in the medium as measured by the absorbance at 490 nm, and it is directly proportional to the number of living cells in culture. For the determination of cell proliferation, $1x10^5$ cells were plated in triplicate on p96 plates in complete medium (10% FBS) to detect the basal proliferation, and also in serum free medium. 48 hours later, 20 µL of CellTiter 96® AQ_{ueous} reagent was

added to the medium. After 1hour and 30 minutes, the absorbance of the plate was measured at 490 nm. Proliferation assays were performed a minimum of three times.

9 Cell migration assays

Cell migration is a highly integrated, multistep process that orchestrates embryonic morphogenesis, tissue repair and regeneration. Errors during this process have severe consequences, including tumour formation and metastasis. In our experiments, we quantified the cell migration rate of each cell line by means of two different cell migration assays: transwell and videomicroscopy.

9.1 Transwell migraton assay

The transwell migration assay is a simple method to study directional cell migration *in vitro*. We used cell culture inserts with a transparent PET (Polyethylene terephthalate) membrane of 8 μm pore size in a 24-well plate. The membrane serves as a barrier to discriminate migratory from non-migratory cells. Migratory cells are able to extend protrusions towards chemoattractants (via actin cytoskeleton reorganization) and ultimately pass through the pores of the PET membrane. These migratory cells are then stained with 0.1% violet cresyl and measured by absorbance.
In our cell migration studies, $2.5x10^5$ H, HG, HGE, HGEshC, HGEshNID1 and HGEshNUPR1 cells were plated on 0% serum media in triplicate and incubated for 48 hours. The cells were fixed by 4% PFA (paraformaldehid) and after washing the non-migratory cells, cells were stained with 1% violet cresyl. The stained membranes were cut and violet cresyl was dissolved with acid acetic 10% dilution and measured by 590 nm absorbance. Migration assays were performed a minimum of three times.

9.2 Videomicroscopy

Videomicroscopy is another method to assess the migration capacity of the cells. It consists in a photography series of the cells. 2.5×10^5 cells in a p6 dish were kept in a computer-controlled miniincubator, which provided a stabilized temperature of 37 +/- 0.5 ºC, with 95% humidity and 5% CO_2, and optical transparency for microscopic observations. The incubator is fastened to an inverted microscope (Live Cell Imaging

CellR, Olympus). Images were taken with the 10x objective every 30 min for 48 h. At least 50 cells per video were manually tracked using the WCIF ImageJ software. Comparisons between cell trajectories from the different cell types and conditions were determined by the maximum relative distance to origin (MRDO) variable.

10 Cell invasion assay

Invasiveness requires several distinct cellular functions including adhesion, motility, detachment and extracellular matrix proteolysis. To elucidate the mechanisms by which tumour cells acquire an invasive phenotype, *in vitro* assays that mimic the *in vivo* process have been developed. For the inverted invasion assay that mimics the physiological and pathological process, the cells should extravasate the pores and invade the matrigel.

Endometrial carcinoma cells were cultured as described (Section 2.1 Endometrial Cancer Cell Lines). Cells were seeded at $5x10^5$ cells/ml directly onto the opposite face of the 8μm size pore membrane transwell (Corning) and incubated for 5 h prior to turning the right side up (Figure 20). Diluted matrigel growth factor-reduced (BD Bioscience) was placed over the upper well. Inserts were placed in a serum-free medium while a 10% FBS complemented medium was applied on top of the matrigel. Complete medium (10% FBS) was used as chemoattractant on the upper face of the insert. Living cells were stained with 4 μM of calcein-acetoxymethyl ester (Invitrogen) and visualized by confocal microscopy after 10 days. Images were scanned at 5 μm intervals using a 10x objective. mRNA extraction from cells invading into the matrigel were extracted and quantified by RTqPCR, using the protocol previously described by Muinelo-Romay in 2011 [270]. Inverted invasion assays were repeated at least three times.

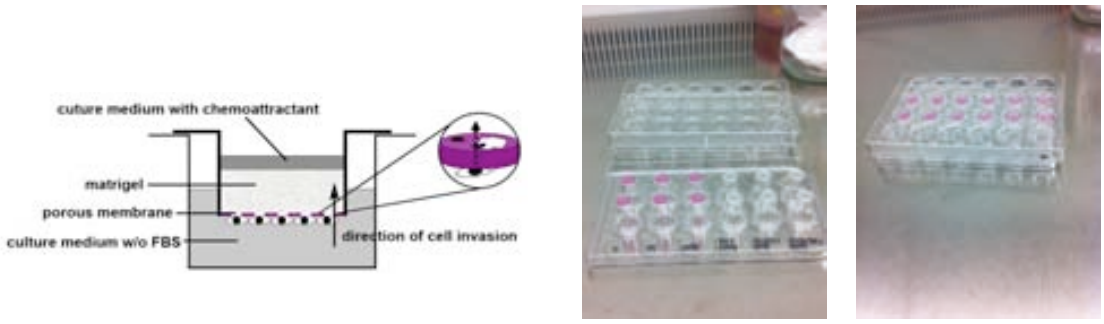


Figure 20. Inverted transwell assay. The first step is to incubate the cells on the bottom side of the transwell membrane for 5 hours.

11 Cell adhesion assay

Cell adhesion is a complex process involved in embryogenesis, migration/invasion, tissue remodelling and wound healing. To carry out these processes, cells adhere to extracellular matrix components via adhesion receptors, forming complexes with components of the cytoskeleton that ultimately affect cell motility, differentiation, proliferation and survival.

$1x10^5$ cells were seeded into laminin- and colagen I-coated coverslips and allowed to adhere between 20 and 40 min depending on the matrix. Adherent cells were fixed and stained with 1:1000 dapi and 1:200 phalloidin. At least five random fields per coverslips were manually quantified using the FSX100 microscope (Olympus). All experiments were done in triplicate. Measurements were made in at least two independent experiments. Student's T-test was performed using SPSS 16.0 software (SPSS, IBM, Armonk, NY, USA).

12 Apoptosis assay

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation.

Cells were plated at 10,000 cells per well in a 96-well plate with 10% FBS medium or serum-free medium in serum deprivation conditions, using three replicates for each

condition: basal, serum deprivation and H_2O_2 apoptosis. Cells were treated during 1 hour before the reading with 1mM H_2O_2. After 24 h, Caspase 3/7 activity was determined according to manufacturer's instructions (Promega). Briefly, 100 µl of Caspase-Glo-3/7 Reagent was added to each well of the 96-well plate containing 100 µl of blank medium. Finally, we measured the luminescence of each sample in a plate-reading luminometer as indicated by the luminometer's manufacturer. Background readings were determined from wells containing culture medium without cells, and mean values resulted from at least three independent experiments.

13 Orthotopic model animal

To better understand the molecular mechanisms of EC and to obtain improved therapies, the use of clinically relevant mouse models, which should include tumour progression, invasion and metastases, is an essential requirement. Orthotopic mouse models of human cancer represent an important *in vivo* tool for drug testing and validation. Our group has recently established an orthotopic mouse model of endometrial cancer which features cancer cells growing in their natural location, and replicates human disease with high accuracy [267]. In this model, endometrial cancer cells are injected into the mouse's uterine cavity where the tumour grows and invades adjacent organs following myometrial invasion.

Six-week old female athymic nude mice (Charles River Laboratories, Inc, Wilmington, MA) were used in this model. The animals were housed in individually ventilated cage units and were maintained under pathogen-free conditions. Food and water were provided *ad libitum*. The animals were sacrificed for necropsy by cervical dislocation after sedation. All the procedures regarding experimentation and animal care were performed according to the guidelines of the Spanish Council for Animal Care and the protocols of the Ethics Committee for Animal Experimentation of our institution. Mice were anesthetized with 2% isofluorane (ABBOT Laboratories, Madrid, Spain), and the lower abdomen was swabbed with iodine. A longitudinal incision (medial laparotomy) was performed and the murine uterus was exposed. 1x 10^6 HGE, HGEshC, HGEshNID1 and HGEshNUPR1 endometrial cells were resuspended in 50 µl of matrigel (BD Matrigel Basement Membrane Matrix, BD

Biosciences, San Jose, CA) and injected directly into the endometrial cavity through the myometrium (Figure 21). A 27 G insulin syringe (Myjector 1 ml, Termo, Somerset, NJ) was used for the injection. The strain on the endometrial cavity and the expulsion of a small quantity of fluid through the vagina ensured the correct location of the injection. Mice were sacrificed seven weeks after the injection. During necropsy, a macroscopic evaluation was performed. Organs were obtained in the following order: axillary lymph nodes, lungs, mediastinal and inguinal lymph nodes, peritoneum, uterus-adnexa and pelvic fat, paraaortic and mesenteric lymph nodes, pancreas, spleen, kidneys, liver and diaphragm. The uteri were photographed by the IVIS-Spectrum (In Vivo Imaging System) to further evaluate and quantify GFP intensity. The fluorescence emitted from the cells was detected, digitalized and electronically displayed as a pseudocolour overlay onto a gray scale animal image.

All tissues were formalin-fixed and processed for routine histological examination. Hematoxilin-Eosin (H&E) staining was performed on 24 h formalin fixed paraffin embedded 4μm sections using routine histological procedures. Histological examination was carried out by an experienced pathologist.

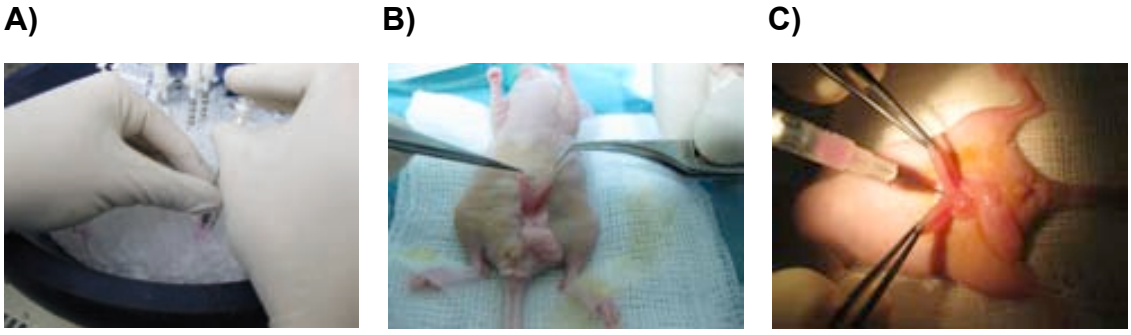


Figure 21. Images of the *in vivo* assay. A) Preparation of the cells mixed with matrigel; the preparation should be on ice to avoid matrigel solidification. **B)** Location of the uterus after the incision. **C)** Injection of the cells into the uterus.

14 Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Science (SPSS, IBM, USA) version 16.0. Student's T-test was used to compare means in mRNA expression levels (microarrays and RTqPCR), Mann-Whitney U test was used to compare means in migration (videomicroscopy and transwell), invasion and

proliferation assays. The non- parametric Spearman's rho test was applied to analyze the correlation between ETV5, NUPR1 and NID1 expression in human tumour samples. T- test was used to compare the percentage of stained cells between invasive and non-invasive tumors. A paired t-test was used to compare the percentage of stained cells between the superficial and the invasive front of the tumor.

RESULTS

1 ETV5 regulated genes in Hec1A endometrial cancer cells.

The gene expression profile of Hec1A endometrial cancer cells with overexpression of GFP-ETV5 (HGE) was compared to Hec1A control cells using microarray technology [137]. Differential gene expression analysis showed that an increase in ETV5 expression was associated with a significant modulation (1.5-fold change, $P<0.001$) of the expression of 225 genes.

The differentially expressed genes (FC<-1.5; >1.5 and an adjusted P <0.01) were exported to the Ingenuity Pathway Analysis (IPA) to determine the biological mechanisms, pathways and most relevant functions of the genes of interest. IPA software uses a knowledge-based database that originates from genes and gene associated functions research output. The choice of networks is based on the random selection of focus genes with maximum connectivity; several interconnected focus genes are clustered as a network and graded from higher to lower scores. Scores are calculated by means of Fisher's exact test, and they represent the ratio between the number of all genes in a specific network against the number of focus genes. Seven networks out of 17 yielded a score over 2. This score indicates the likelihood that the assembly of a set of genes in a network could be explained by random chance alone. Networks with scores of 2 or higher have a minimum confidence of 99% of not resulting from random chance alone. The network identified with the highest score was "Cancer, Gastrointestinal Disease, Inflammatory Response". All the significant gene networks are included in Table 12.

Table 12. List of significant networks of genes associated with differentially expressed genes in Hec1A cancer cells overexpressing ETV5.

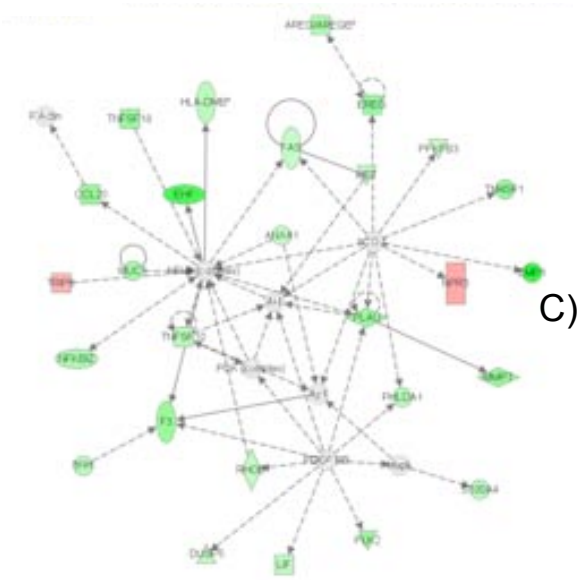
ID	Molecules in network	Score	Focus mol	Top functions
1	*Akt,ANXA1,Ap1,AREG/AREGB,CCL20,DUSP5,EHF,EMP1,EREG,F3,FActin,FAS,hCG,HLA-DMB,LIF,Mapk,MET,MMP7,MUC1,NFkB(complex),NFKBIZ,NPR3,PDGFBB,PFKFB3,PHLDA1,PI3K(complex),PLAU,PLK2,RHOB,S100A4,TFPI,TM4SF1,TNFSF10,TNFSF18,TRIP6*	42	27	**Cancer**, Gastrointestinal Disease, Inflammatory Response
2	*Actin,Caspase,CCND1,CDH1,CLDN4,ERK,ERK1/2,FGFBP1,FLNA,Focal adhesion kinase,GPRC5A,Growthhormone,Histoneh3,ICAM1,IFI35,IGFBP3,IgG,ITGB3,ITGB6,Jnk,KITLG,KLK6,KRT8,KRT13,KRT16,KRT19,P38 MAPK,PDZK1,PPL,PRSS8,RNA polymerase II,SERPINA3,SFN,Tgf beta,TNC*	33	23	**Cell-To-Cell Signalling** and Interaction, Connective Tissue Development and Function, Tissue Development
3	*AKR1C1/AKR1C2,Cbp,CCNE2,CDH2,CLDN4,CLDN16,CTH,ETV5,GADD45B,HSPB8,IFI16,MAP2K6,mir-21,MMP2,NAP1L3,P38 MAPK,PDGFRB,PLK2,PRDM5,PTK2,PTP4A3,RB1CC1,S100A4,SERPINB5,SFN,TGFB1,TINAGL1,TNC,TP53,TP73,TPM1,TSPAN1,TSPAN7,ZEB1,ZFP36L1*	23	18	**Cell Cycle**, **Cancer**, Reproductive System Disease
4	*CASP1,CASP6,CCL28,CD24,CD209,CEACAM1(includes others),CSF2,CXCL1,CXCL5,CYBB,EIF4B,ELF3,ERBB2,ERRFI1,FAM65B,FGB,GAL3ST1,IFNG,IGFBP2,KLK10,KRT7,LAMB3,LGALS3BP,lymphotoxin-alpha1-beta2,MAL,NID1,PIGR,SATB1,SEMA3C,TLR5,TMSB10/TMSB4X,TNF,TRIM22,VCAM1,WNT7A*	18	15	**Cellular Movement**, Haematological System Development and Function, Immune Cell Trafficking
5	*ACTN1,ADAMTS1,AHNAK2,AMIGO2,APOL1,AREG/AREGB,ARL4C,BAD,BMPR1B,CFLAR,CREB5,CTNND1,CXCR4,CYP19A1,DAPK1,DHRS3,EREG,FSH,HIC1,IGF2BP1,ILK,KRT18,LDLR,Lh,MAP1LC3B,MAP2K3,NPC2,PRKD1,PTPRF,RHOB,SGK1,TFPI2,TNFRSF11B,TPM1,VCL*	14	13	DNA Replication, Recombination, and Repair, **Cancer**, **Cell Death**
6	*ACSL1,BIRC5,CDKN1B,CEBPA,CEBPB,CYP2B6,ESR1,FGD6,FOXA2,FOXO3,GNB2L1,GPAM,HAVCR1,HNF1A,HNF4A,ICAM2,ID2,ITGAL,MUC4,NR0B2,NR1I2,NR1I3,NUPR1,PHLDB2,PIM1,PLOD2,RXRA,SECTM1,SERPINA1,SGK1,SMAD3,TM4SF4,TMOD1,VDR,YWHAG*	14	13	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
7	*AHNAK,ANGPT2,ARHGDIA,ARHGDIB,CDK2,CDKN2A,CLRN1,E2f,EFNA5,Estrogen Receptor,FN1,HIST1H2BJ/HIST1H2BK,HIST1H3A (includes others),HMGA1,IFN Beta,INSIG1,ITGA5,ITGB8,KDR,LGALS3,NRP1,PDP1,PRKCA,RB1,RBL1,RBL2,REST,RHOA,SERPINE2,SLC16A1,SMARCA4,SMARCB1,STK11,TFDP1,YY1*	13	12	Cell Morphology, Cellular Development, **Cell Cycle**

Transcripts that were differentially regulated were also subjected to a pathways and biological functions analysis using the IPA software. D analysis, IPA finds the assigned function for specific genes and further c genes with related biological functions into biofunction classes. The top thre classes were grouped by the IPA software into Disease and Disorders, Mol Cellular Functions and Physiological System Development and Funct biological functions most represented in each group were cancer, cellular and tissue development, respectively. The most significant canonical represented was "*ILK* signalling".

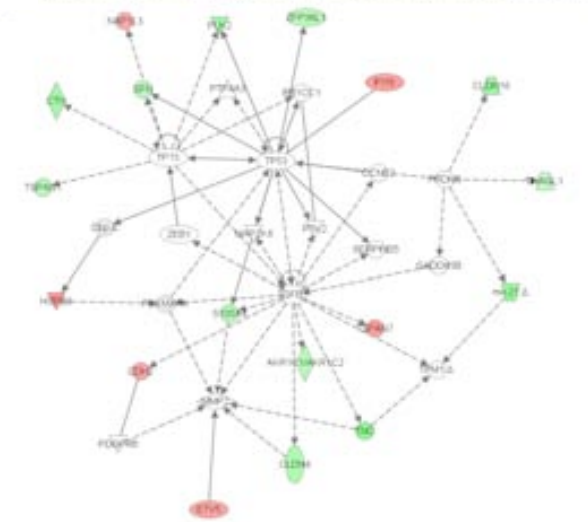
Interestingly, analysis of the molecules and connections included in the top pointed to *CDH1* (E-cadherin) and *ERK1/2* in Network 2 and *TGFβ*, *TP53* a in Network 3 as the signalling pathways altered when under control by ETV 22). These results support our previous findings related to ETV5 fu endometrial cancer. We have already demonstrated that the cell adhesion m cadherin is regulated by ETV5 through the induction of the ZEB1 represso addition, it has been shown that the *TGFβ* signalling pathway has been mo ETV5 downregulation in OV90 ovarian cancer cells [209]. Also, it demonstrated that *MMP2* is regulated by ETV5 and involved in the in endometrial cancer cells *in vitro* and *in vivo* [148]. Regarding Network1 molecules are *NFKB* and *AKT*. Both signalling pathways have been in survival of tumour cells. Future work will examine the possibility of the *I AKT* pathways being modulated by ETV5 overexpression.

A)

B)



C)



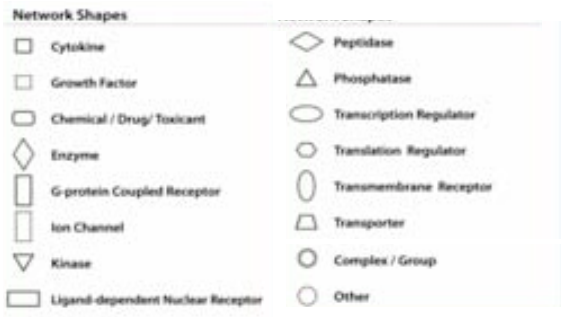

Network Shapes
Cytokine
Growth Factor
Chemical / Drug/ Toxicant
Enzyme
G-protein Coupled Receptor
Ion Channel
Kinase
Ligand-dependent Nuclear Receptor
Peptidase
Phosphatase
Transcription Regulator
Translation Regulator
Transmembrane Receptor
Transporter
Complex / Group
Other

Figure 22. IPA gene network analysis of differentially expressed genes between Hec1A and Hec1A GFP-ETV5 cells. Intensity of the red (up-regulated) or green (down-regulated) colour indicates the level of gene expression. White indicates a predicted molecule incorporated from the Ingenuity knowledge base. A line represents the binding of proteins, whereas a line with an arrow represents "act on". A dotted line represents an indirect interaction. **A)** The top network included 27 differentially expressed molecules with *NFKB* and *AKT* as the core molecules of the network. **B)** The second top network included 23 differentially expressed molecules with *CDH1* (E-cadherin), and *ERK1/2* as the core molecules of the network. **C)** The third top network included 18 differentially expressed molecules with *TGFβ*, *TP53* and *MMP2* as the core molecules of the network.

In order to identify ETV5 target genes involved in tumour invasion, we selected for further analysis seven upregulated genes in Hec1A cells overexpressing ETV5 (Table 13). This selection was based on fold-change and statistical significance (FC over 1.3 and P <0.05), the presence of putative ETS binding sites in their promoter regions and a suspected role in cancer invasion (*ANGPT-2, PLOD-2, NUPR1, EFNA5, NPR3, NID1* and *LAMP3*).

Typically, promoter regions are immediately adjacent to the gene and positions in the promoter are designated in relation to the transcriptional starting site. We searched for putative ETS binding sites (5′- GGAA/T-3′) in the 1000 base pairs 5' to the transcription starting site. We also searched for other regulatory transcriptional sequences such as TATA box (5'-TATAAA-3'), SP1 (5'-CCGCC-3') and AP1 elements (5'-TGAGTCA-3') to verify that we were analyzing the promoter region.

In HGE cells we identified a number of genes significantly upregulated (>1.3 fold, P<0.05): *BMPR1B, SERPINE2, FOLH1B, CDH2, FOLH1, CDK6, BIRC7*. Most of these genes have been described in tumour progression in different types of cancer. However, we decided not to analyze those genes where we could not identify a reliable promoter region on its 1000 base pairs 5' to the transcription starting site.

As expected, *ETV5* appears upregulated with a fold change of almost 2, as well as *ICAM-1*, which has been already described as directly regulated by the *ETV5* transcription factor [271].

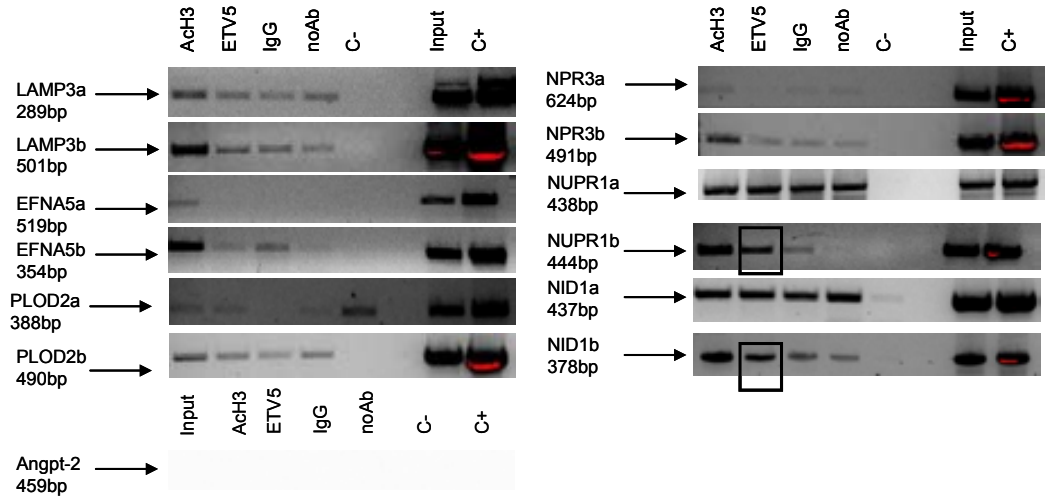
Table 13. Summary of the genes modulated by ETV5 in the microarray assay. The seven selected genes are underlined in green. Gene selection was based on the fold change, ETS binding sites in their promoter and a putative role in tumour progression.

Symbol	Entrez Gene Name	p-value	Fold Chg
CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6	5,10E-16	5,06830513
BMPR1B	bone morphogenetic protein receptor, type IB	9,16E-17	3,61813172
DKFZp686O24166		1,50E-13	3,4421192
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	8,64E-17	3,16526154
ANGPT2	angiopoietin 2	8,77E-15	3,18148719
NUPR1	nuclear protein, transcriptional regulator, 1	3,31E-14	2,80059159
SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	7,54E-16	2,59079947
C18orf19	chromosome 18 open reading frame 19	7,34E-12	2,55831426
NFIA	nuclear factor I/A	1,12E-13	2,41906714
CNTN4	contactin 4	1,43E-11	2,31793924
TEX15	testis expressed 15	3,73E-08	2,29350537
EFNA5	ephrin-A5	9,33E-10	2,33677419
HSPB8	heat shock 22kDa protein 8	8,72E-12	2,29284644
MPDZ	multiple PDZ domain protein	3,91E-10	2,21259852
LOC728212	hypothetical LOC728212	1,06E-08	2,13268826
LYST	lysosomal trafficking regulator	6,43E-11	2,20050468
TTYH3	tweety homolog 3 (Drosophila)	5,78E-14	2,12336247
LOC728212	hypothetical LOC728212	4,41E-08	1,94093484
LOC728212	hypothetical LOC728212	7,26E-09	2,00399353
TSPAN7	tetraspanin 7	1,79E-11	2,02812977
SERPINE2	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	3,82E-12	2,02378228
MTUS1	microtubule associated tumor suppressor 1	3,97E-09	2,01750099
IFI16	interferon, gamma-inducible protein 16	4,00E-10	1,95679486
FLJ26056	hypothetical protein LOC375127	6,00E-09	1,90133332
SATB1	SATB homeobox 1	2,27E-11	1,97480899
FOLH1B	folate hydrolase 1B	1,88E-10	1,85821335
CDGAP	Rho GTPase activating protein 31	1,24E-10	1,8730086
ETV5	ets variant 5	1,98E-12	1,93163908
CDH2	cadherin 2, type 1, N-cadherin (neuronal)	2,80E-12	1,85674924
FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	1,22E-10	1,81033416
NPR3	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	1,09E-12	1,78402339
MCTP1	multiple C2 domains, transmembrane 1	1,04E-11	1,82716336
NAP1L3	nucleosome assembly protein 1-like 3	6,87E-10	1,81692158
NID1	nidogen 1	1,74E-11	1,80873287
LOC25845	hypothetical LOC25845	2,11E-11	1,72485245
FLJ26056	hypothetical protein LOC375127	1,98E-07	1,64985815
FLJ26056	hypothetical protein LOC375127	1,98E-07	1,64985815
EMID2	EMI domain containing 2	3,43E-09	1,63933874
CWH43	cell wall biogenesis 43 C-terminal homolog (S. cerevisiae)	2,88E-12	1,64138057
LOC728212	hypothetical LOC728212	5,55E-09	1,68221155
JPH1	junctophilin 1	5,90E-08	1,7094977
APOC1	apolipoprotein C-I	1,88E-10	1,70687871
HIST1H2BK	histone cluster 1, H2bk	1,76E-12	1,70470976
ICAM1	intercellular adhesion molecule 1	6,13E-06	1,68695716
PCDH24	cadherin-related family member 2	4,03E-10	1,63832951
LOC728212	hypothetical LOC728212	5,55E-09	1,68221155
FLJ26056	hypothetical protein LOC375127	1,98E-07	1,64985815
FLJ26056	hypothetical protein LOC375127	1,98E-07	1,64985815
CWH43	cell wall biogenesis 43 C-terminal homolog (S. cerevisiae)	2,88E-12	1,64138057
EMID2	EMI domain containing 2	3,43E-09	1,63933874
DGKH	diacylglycerol kinase, eta	1,20E-11	1,62324072
IGF2BP1	insulin-like growth factor 2 mRNA binding protein 1	2,10E-10	1,60842788
CYP2J2	cytochrome P450, family 2, subfamily J, polypeptide 2	8,95E-10	1,33794636
RNMT	RNA (guanine-7-) methyltransferase	2,22E-07	1,29670691
CDK6	cyclin-dependent kinase 6	5,02E-09	1,2950852
CT45A1	cancer/testis antigen family 45, member A1	2,33E-07	1,36560822
DDR2	iscoidin domain receptor tyrosine kinase 2	0,00018295	1,31222367
BIRC7	baculoviral IAP repeat containing 7	1,00E-07	1,31377666
FN1	fibronectin 1	2,20E-06	1,31768141
LAMP3	lysosomal-associated membrane protein 3	2,89E-09	1,3278793

Primers were designed to amplify the ETS binding sites located in the gene promoters (See Table 11 in Section 6 ChIP). The selected genes were analyzed by chromatin immunoprecipitation (ChIP) to determine whether ETV5 interacted with their promoters.

The results of ChIP analysis demonstrated that ETV5 binds to the proximal promoter region of Nidogen 1 (*NID1*) and *NUPR1* (Figure 23), suggesting that both genes may be direct ETV5 transcriptional targets.

A)



B)

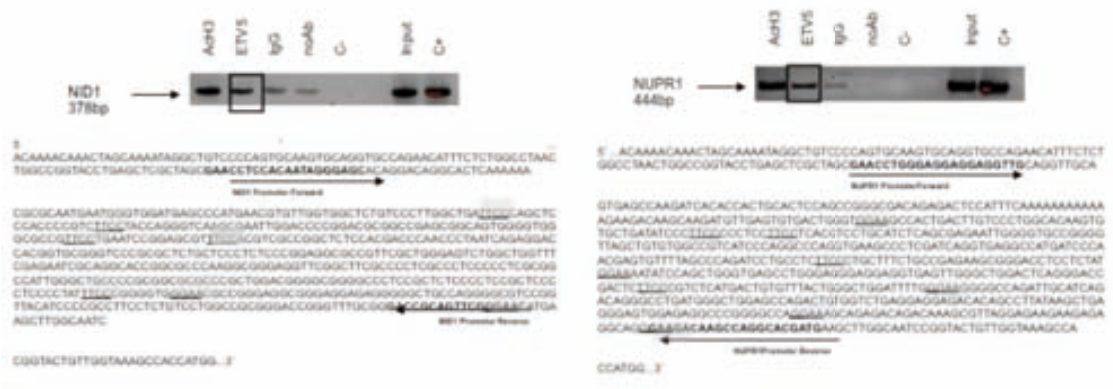


Figure 23. ChIP analysis of ETV5 binding to the *LAMP3, CD44, EFNA5, ANGPT-2, PLOD2, NPR3, NUPR1* and *NID1* promoters. A) Some promoter regions have been amplified in two fragments ("a" and "b") to cover all putative ETS binding sites. Irrelevant IgGs and negative controls, and antibodies directed against acetylated histone are shown as negative and positive controls, respectively. **B**) Representation of NID1 and NUPR1 promoter

region shows seven and eight ETS, indicating putative binding sites 5' to the transcriptional start site (TSS) (in insets). Underlined, forward and reverse primers for the promoter region comprising nucleotides from -375 to 3 for *NID1* and from -444 to -30 for *NUPR1*.

NID1 is a glycoprotein located in the basement membrane, where it interacts with several components of the extracellular matrix, more specifically with laminin and collagen IV [248]. The major biological functions of NID1 involve extracellular matrix assembly, homeostasis and wound healing, enhancement of cell attachment and enhancement of chemotaxis and phagocytosis [253]. On the other hand, NUPR1, also known as p8 and COM-1 (candidate of metastasis-1), is a small protein related to the high mobility group of transcriptional regulators [242]. This protein is involved in different biological functions such as endoplasmic-reticulum response, programmed cell death, control of gene expression by regulation of chromatin accessibility and regulation of cell cycle progression [223].

In order to prove that ETV5 is a transcriptional activator of *NID1* and *NUPR1*, we cloned the *NID1* and *NUPR1* promoter regions containing the ETS binding sites into a luciferase reporter gene construct. We found an increase in luciferase expression using both promoter regions, indicating that ETV5 directly regulates both genes at the transcriptional level.

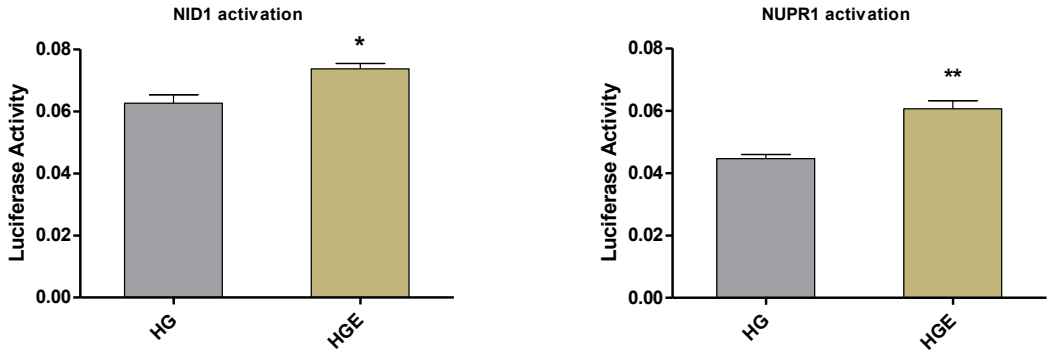


Figure 24. ETV5 acts as transcriptional inducer of *NID1* and *NUPR1*. Hec1A GFP and Hec1A GFP-ETV5 cells were transiently co-transfected with the pGL4.15 luciferase reporter vector cloned with the *NID1* and *NUPR1* promoter region comprising nucleotides from -375 to 3 and from -444 to -30, respectively, to the TSS. Relative luciferase activity is shown. Error

bars represent mean ±sd. of duplicate experiments (Student's T test comparing control cell lines against ETV5 overexpression, *P= 0.041 and **P=0.0016).

Finally, the upregulation of both *NID1* and *NUPR1* in Hec1A cells overexpressing ETV5 was further validated by real time quantitative PCR and Western Blot, verifying that the upregulation of NID1 and NUPR1 happened simultaneously with the upregulation of ETV5 in the Hec1A cell line (Figure 25). Due to the lack of an adequate commercial antibody to use in Western blot, NUPR1 could not be detected at the protein level.

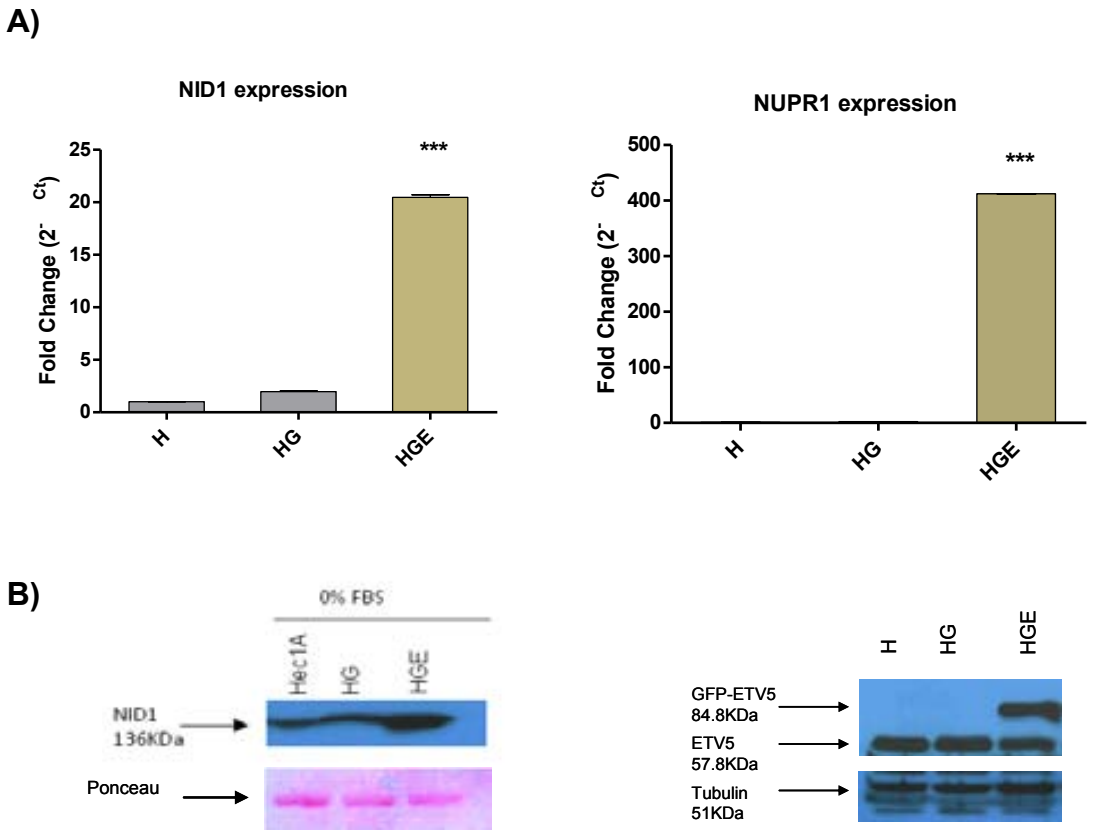


Figure 25. NID1 and NUPR1 are upregulated in Hec1A cells with ETV5 overexpression. **A)** *18S,* a housekeeping gene, was used to normalize mRNA levels in the analysis of *NID1* and *NUPR1* mRNA levels by RTqPCR. Student's T test was used to compare control cell lines against cells overexpressing ETV5 (***P= 0.0001). **B)** Analysis of NID1, endogenous ETV5 and GFP-ETV5 protein levels by WB. NID1 WB was performed on media secreted proteins. ETV5 WB was performed on total cell extract.

To verify that the increased levels of ETV5 are associated with NID1 and NUPR1 upregulation, we checked the mRNA expression levels of *NID1* and *NUPR1* in an additional endometrial cell line transiently transfected with GFP-ETV5. Similar results were obtained in the endometrial cancer cell line AN3CA (Figure 26).

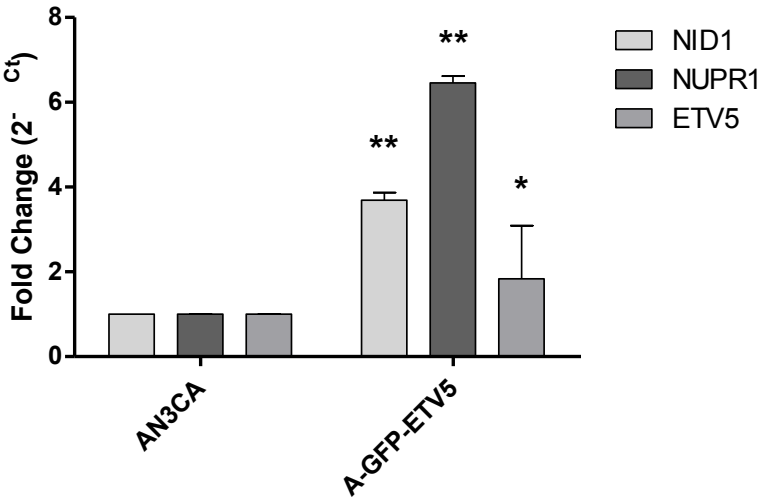


Figure 26. *NID1* and *NUPR1* are upregulated in AN3CA cells with ETV5 transient overexpression. *GAPDH,* a housekeeping gene, was used to normalize mRNA levels in the analysis of *NID1* and *NUPR1* mRNA levels by RTqPCR. Student's T-test was used to compare control cell lines with cells overexpressing *ETV5* (*P=0.05, **P= 0.001).

2 *In vitro* effects of knocking down NID1 and NUPR1 in Hec1A endometrial cancer cells overexpressing ETV5.

Previous work carried out by our group on ETV5 function in endometrial cancer showed that ETV5 overexpression in Hec1A endometrial cancer cells promoted cell scattering, cell adhesion at different matrices and cell migration and invasion both *in vitro* and *in vivo* [137, 272].

In order to study the putative role of NID1 and NUPR1 in ETV5 dependent migration and invasion, we firstly knocked down *NID1* and *NUPR1* in Hec1A cells overexpressing GFP-ETV5 (HGE). HGE cells were transduced with three different short hairpin sequences ("a", "b" and "c") to assess the best working short hairpin against both genes using lentiviral constructs. To assess the effectiveness of shRNA in knocking down *NID1* or *NUPR1* expression, their mRNA and protein levels were examined by RTqPCR and Western blot, respectively. The reduction in *NID1* and *NUPR1* expression mRNA levels was approximately 86% for NID1 and 91% for NUPR1 in HGEshNID1 and HGEshNUPR1 cells, compared to HGEsh control cells (Figure 27A). We selected the mix population with higher inhibition of *NID1* or *NUPR1* at mRNA level and with *ETV5* mRNA levels not affected (HGEshNID1a and HGEshNUPR1b, respectively) (Figure 27B).

The protein levels of NID1, NUPR1 and ETV5 were examined to confirm the reduction of protein expression of both genes in the selected HGEshNID1a and HGEshNUPR1b cells. We also checked that ETV5 protein levels had not been affected in HGE cells (Figure 27C). NID1 protein levels were completely reduced in HGEshNID1a cells while ETV5 levels were not affected (Figure 27C). Again, NUPR1 could not be detected at the protein level. We finally chose HGEshNID1a and HGEshNUPR1b modified cells for our functional assays.

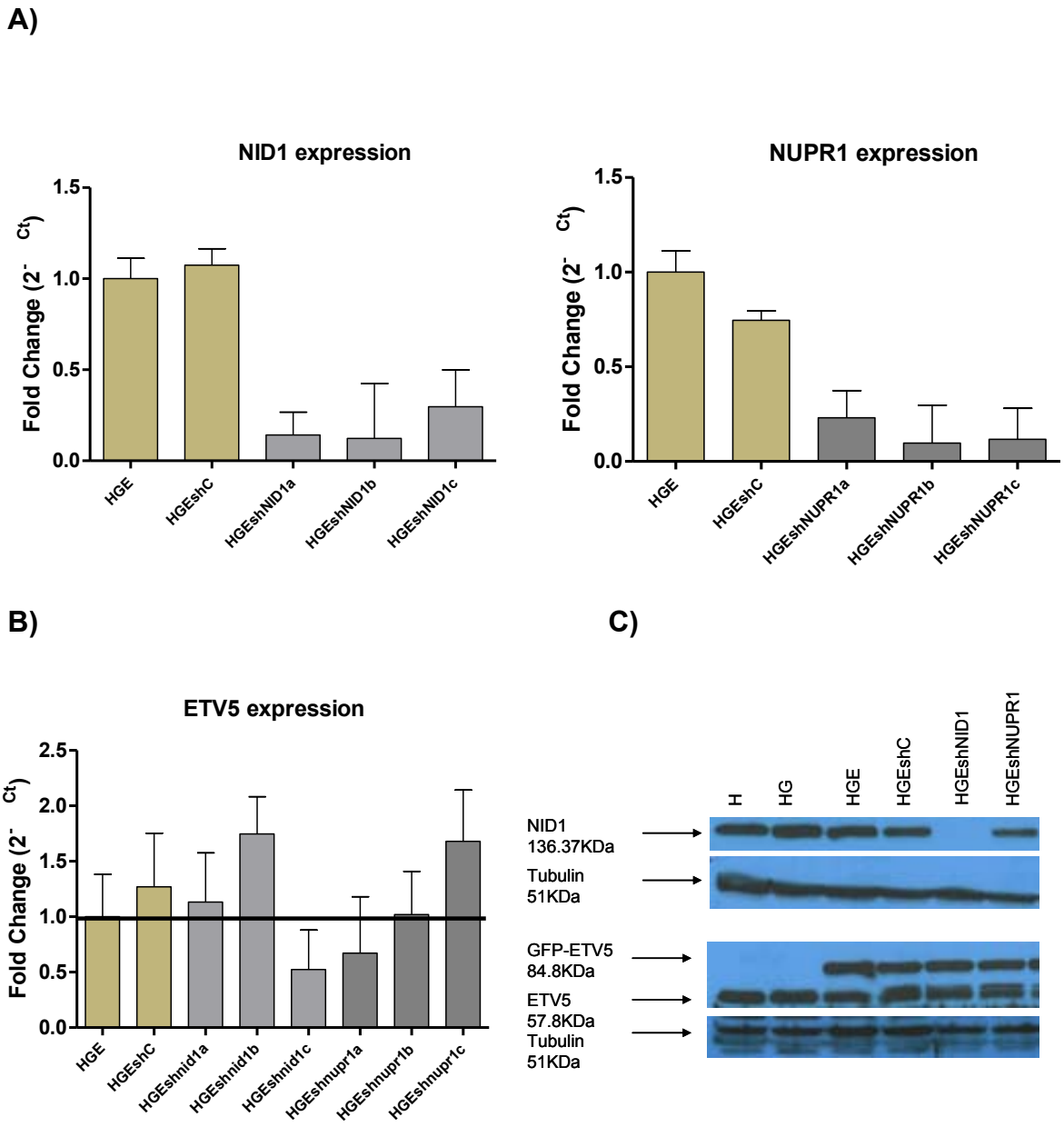


Figure 27. HGE cells were transduced with stable short hairpins lentiviral constructs in order to block *NID1* and *NUPR1* expression. **A** and **B)** To assess the best working short hairpin sequences, we performed RTqPCR to examine the expression of *NID1, NUPR1* and *ETV5* mRNA levels in HGEsh transduced cells. *18S*, a housekeeping gene, was used to normalize mRNA levels. **C**) We validated the inhibition of NID1 at protein level by WB analyses and the no modification of ETV5 expression in all cell lines.

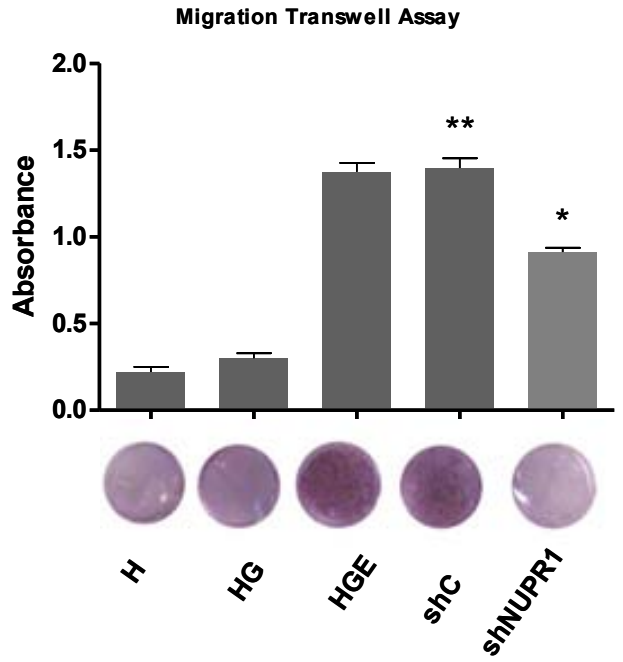
The effects on *in vitro* cell functions by NID1 and NUPR1 were examined. First, we assessed changes in cell migration, invasion and proliferation in HGE cells with NID1

or NUPR1 downregulation. HGE cells with NID1 downregulation showed reduced cellular invasion using an inverted cell invasion assay [270]. However, inhibition of NID1 did not affect cell migration. In contrast, HGE cells with NUPR1 downregulation exhibited reduced cell migration, as measured both by transwell assay and videomicroscopy, but they failed to show reduced cell invasion (Figure 28).

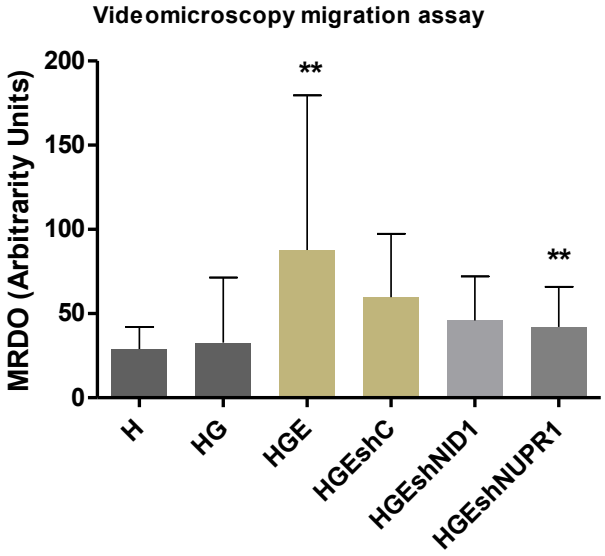
See next page;

Figure 28. Inhibition of NID1 and NUPR1 in Hec1A endometrial cancer cells with GFP-ETV5 overexpression reduces cell migration and invasion *in vitro*. A) and **B)** Cell migration was assessed both by a migration transwell and a videomicroscopy assay on HGE cells with NID1 or NUPR1 downregulation. Hec1A, Hec1A GFP cells and HGEshC cells were used as controls. **C)** The inverted cell invasion assay showed a reduction of the invasion capacities of HGEshNID1 cells through matrigel. Calcein was used to stain the cells in the matrigel. The cells were then analyzed by serial slides in a confocal microscopy. Mann Whitney´s test was used to analyse the three assays (*P<= 0.05).

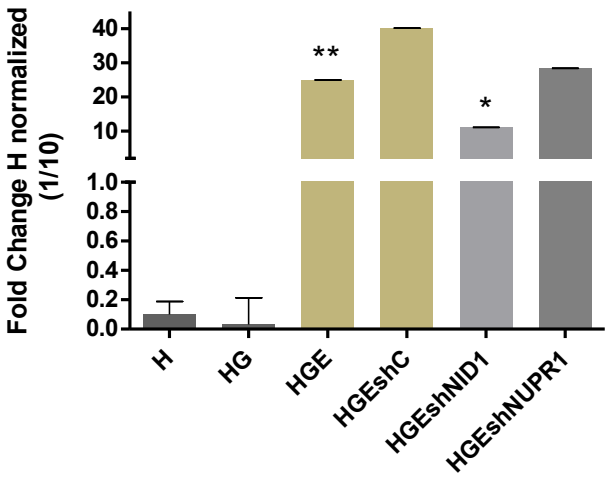
A)


Migration Transwell Assay
Absorbance
2.0
1.5
1.0
0.5
0.0
**
*
H
HG
HGE
shC
shNUPR1

B)


Videomicroscopy migration assay
MRDO (Arbitrarity Units)
200
150
100
50
0
**
**
H
HG
HGE
HGEshC
HGEshNID1
HGEshNUPR1

C)


Fold Change H normalized (1/10)
40
30
20
10
1.0
0.8
0.6
0.4
0.2
0.0
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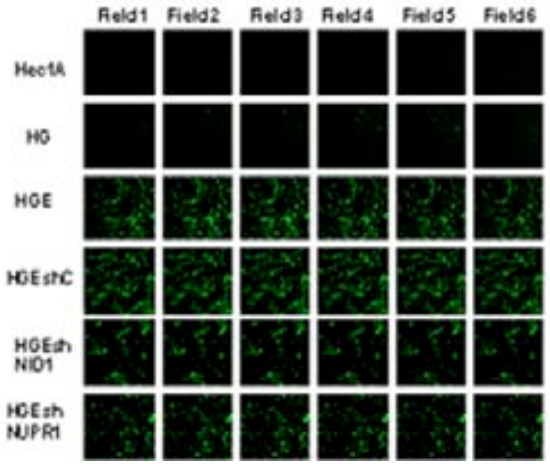
H
HG
HGE
HGEshC
HGEshNID1
HGEshNUPR1


Field1
Field2
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Hec1A
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HGEsh NID1
HGEsh NUPR1

Finally, we examined the effect of reducing NID1 and NUPR1 on cell proli
the presence and absence of serum after 48 hours. No changes were s
proliferation capacities of HGEshNID1 or HGEshNUPR1 cells compared to

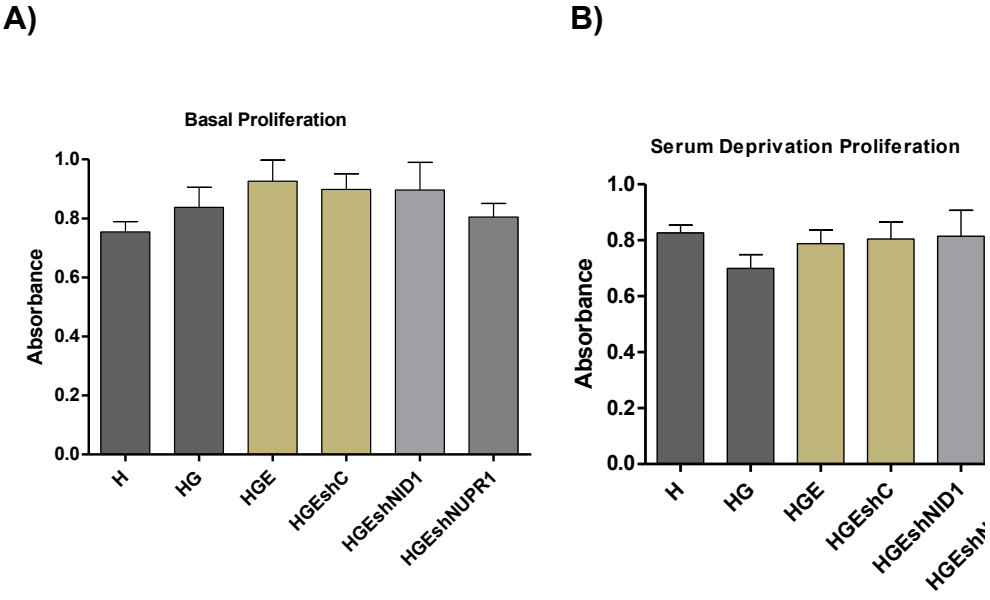


Figure 29. Inhibition of NID1 and NUPR1 in Hec1A cells overexpressing ETV5 affect cell proliferation. HGEshNID1 and HGEshNUPR1 cells and controls were complete medium **A)** and serum-free medium **B)** and allowed to grow for 48 h.

Previous work has shown that Nidogen 1 can bind to laminin and collage high affinity. Moreover, we have previously reported that overexpression o Hec1A cells increases cell adhesion to laminin and collagen I. For this r checked the adhesion properties of HGEshNID1 cells to laminin and compared to HGEshC control cells. The results obtained show that ac laminin is reduced in HGEshNID1 cells (Figure 30), suggesting that the adhesion properties to laminin of Hec1A cells with ETV5 overexpressio mediated by NID1.

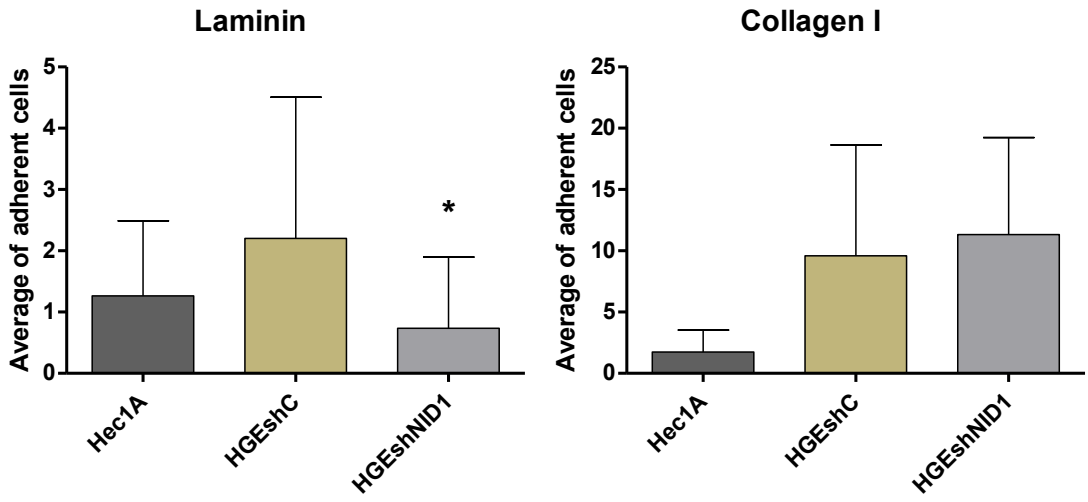


Figure 30. Inhibition of NID1 in HGE cells reduces cell attachment to laminin. Hec1A cells, HGEsh control and HGEshNID1 were plated onto coverslips coated with collagen I and laminin. Quantification of 15 fields is plotted on bar graphs showing media and sd of two independent experiments (* P<0.05).

NUPR1 has been involved in the stress response and in cancer progression. Hec1A cells with ETV5 overexpression have been shown to generate oxidative stress as a consequence of increased migration and invasion [273]. In order to check whether NUPR1 had a protective role against apoptosis induced by oxidative stress, we measured apoptosis induction in HGEshNUPR1 cells and controls quantified by a caspase-activity assay, in basal conditions and after treatment with H_2O_2.

We found that in basal conditions no change in apoptosis between cells was observed, whereas after H_2O_2 treatment cell apoptosis was reduced in cells with NUPR1 inhibition.

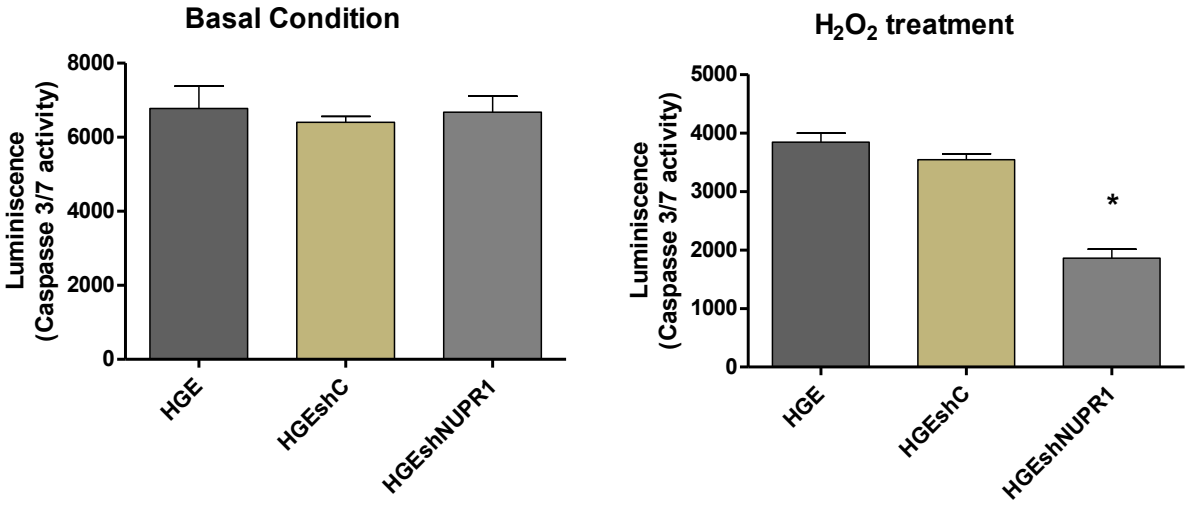


Figure 31. NUPR1 inhibition reduces apoptosis induction after H_2O_2 treatment. Histogram showing the relative apoptosis induction measured as luminescence caspase 3/7 activities (* P<0.05).

To understand the mechanism underlying the effect of NID1 and NUPR1 in cell invasion and migration, we analysed the expression of several EMT markers in HGEshNID1 and HGEshNUPR1 cells. We have previously shown that overexpression of ETV5 in Hec1A cells induces the expression of the *ZEB1* repressor, the loss of E-cadherin and the induction of N-cadherin expression. These changes are concomitant to an increase in cell migration and invasion in Hec1A cells overexpressing ETV5 [137]. Interestingly, we observed that the increase in the expression of N-cadherin in HGE cells was reverted in HGEshNID1 and HGEshNUPR1 cells (Figure 32). In contrast, the expression of E-cadherin was maintained in all four ETV5 overexpressed cell lines (Figure 32).

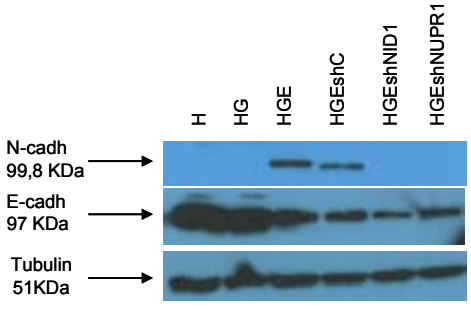


Figure 32. N-cadherin expression is lost in HGEshNID1 and HGEshNUPR1 cells. WB analysis of N-cadherin and E-cadherin expression in HGEshNID1 and HGEshNUPR1 cells and controls.

3 *In vivo* effects of knocking down NID1 and NUPR1 in Hec1A endometrial cancer cells overexpressing ETV5.

To analyze the effects of inhibiting NID1 and NUPR1 in the invasion properties of Hec1A cells overexpressing ETV5 *in viv*o, we injected HGEshNID1, HGEshNUPR1 or HGEshC endometrial cancer cells in the uterus of Swiss nude mice to generate orthotopic mouse models that mimic endometrial tumour myometrial invasion and dissemination [267]. Fifteen nude mice were inoculated with $1x10^6$ HGEshC, HGEshNID1 or HGEshNUPR1 cells in order to evaluate the invasion and migration capacities of NID1 and NUPR1 genes *in vivo*.

We first discarded those mice that developed peritoneal metastases with no tumour growth in the uterus, since it meant that the tumour cell injection in the uterus had not been performed correctly (3 mice in the HGEshNID1 group and 2 mice in the HGEshNUPR1 group). In the HGEshC group, ten of the 15 mice (66.6%) developed endometrial tumours, whereas seven of 12 mice (58.3%) and twelve of 13 mice (92.3%) in the HGEshNID1 and HGEshNUPR1 groups, respectively, developed endometrial tumours. Metastases were observed in 50% (5/10) HGEsh mice, 71% (5/7) HGEshNID1 mice and in 58% (7/12) HGEshNUPR1 mice. Differences of tumour incidences between the groups were not significant. We considered that the differences observed could be attributed to the technical procedure and the small sample size.

We observed peritoneal implants in the pelvic peritoneum, prevesical fat and abdominal implants affecting the pancreas, liver, spleen, kidney, diaphragm and abdominal peritoneum in 5/ 5 HGEshC mice. Lymphatic dissemination was observed in 4/ 5 mice with metastases. Paraaortic lumbar lymph nodes were affected in 3/ 5 mice with metastases; other affected lymph nodes were paraaortic renal (2/5), mesenteric (2/5) and inguinal (2/5). Haematogenous metastases in the lungs were detected in 4/5 animals, but no intrahepatic metastases were observed in our series. All observed metastases were verified by H&E staining.

4 of 5 mice with metastases in the HGEshNID1 group presented peritoneal implants in the pelvic peritoneum, prevesical fat and abdominal implants affecting the pancreas,

kidney, diaphragm and abdominal peritoneum. Lymphatic dissemination was observed in 3/ 5 mice with metastases. Paraaortic lumbar lymph nodes were affected in 3/5 mice with metastases; the inguinal lymph nodes were also affected (2/5). Haematogenous metastases in the lungs were detected in 1 of 5 animals, but no intrahepatic metastases were observed in our series.

Finally, 7 out of 7 mice with metastases of the HGEshNUPR1 group presented peritoneal implants in the pelvic peritoneum, prevesical fat and abdominal implants affecting the pancreas, liver, kidney, diaphragm and abdominal peritoneum. Lymphatic dissemination was observed in 4/7 mice with metastases. Paraaortic lumbar lymph nodes were affected in 5/ 7 mice with metastases; other affected lymph nodes were the paraaortic renal (1/5), mesenteric (2/5) and axillary (1/5). Haematogenous metastases in the lungs were detected in 1/ 5 animals, but no intrahepatic metastases were observed in our series. The incidence of metastases was not significantly different between the groups. Table 14 summarizes the orthotopic endometrial primary tumours and the metastases generated.

Table 14. Comparative results of orthotopic murine models derived from HGEshC, HGEshNID1 and HGEshNUPR1 cells.

	HGEshC	HGEshNID1	HGEshNUPR1
Mice used, n	15	12	13
Mice with endometrial tumour	10	7	12
Tumour incidence, %	66,60%	58,30%	92,30%
Metastases incidence	5/10	5/7	7/12
Peritoneal metastases (pelvic implants and abdominal implants)	5/5	4/5	7/7
Lymph node metastases	4/5	3/5	4/7
Lung metastses	4/5	1/5	1/7

We used the IVIS system to detect the fluorescence of the tumours. The fluorescence emitted from the cells was detected by the IVIS-Spectrum, digitalized and electronically displayed as a pseudo colour overlay onto a gray scale animal image.

Regions of interest (ROIs) from displayed images were drawn automatically around the primary tumour fluorescent signals and quantified as calibrated Radiant Efficiency units. The manual ROIs were drawn along time using the same ROI parameters. We analysed the tumour distribution and quantified the tumour size using the GFP fluorescence signal detected by the IVIS system (Figure 34).

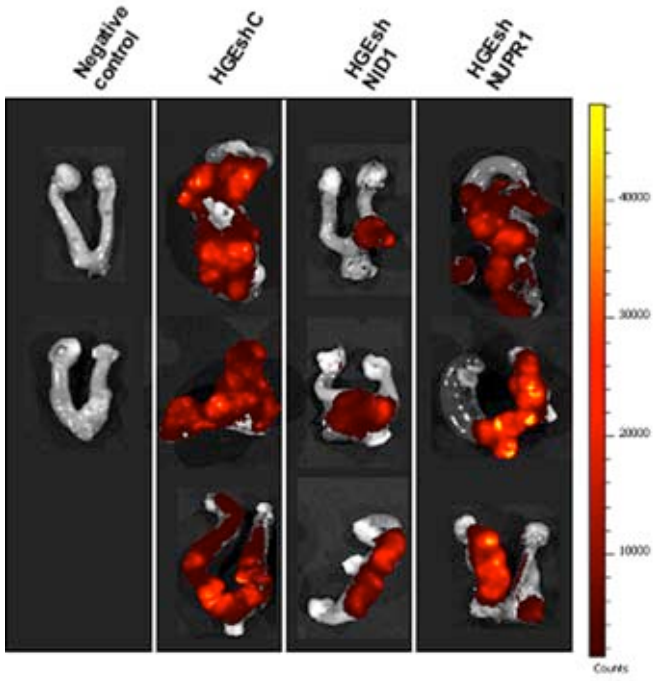


Figure 33. The uteri of the mice were photographed by the IVIS system to detect the GFP fluorescence of the tumours. The uteri were photographed by the IVIS system to quantify GFP fluorescence. Three representative primary uterine tumours corresponded to the three groups of mice. We used as a negative control a uterus from a mouse that had been injected with matrigel alone.

Mice injected with HGEshNID1 cells developed smaller primary tumours (as measured by GFP fluorescence) than control mice (* P= 0.027, Mann Whitney test) and HGEshNUPR1 mice (Figure 34).

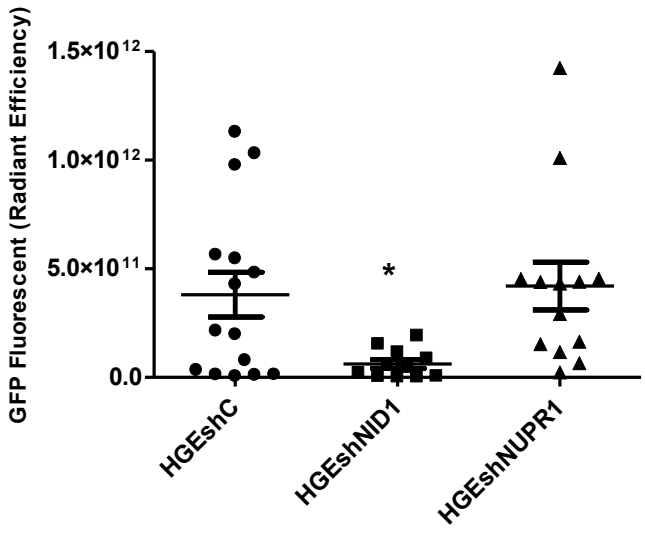


Figure 34. Quantification of uterine primary tumour growth by GFP fluorescence. We used the Living Image 4.0 software to quantify GFP fluorescence. Radiant efficiency units were used to measure GFP intensity in labelled ROIs (regions of interest). Primary tumours from HGEshNID1 cells showed less GFP fluorescence (*P= 0.027, Mann Whitney test).

In order to demonstrate that primary tumours and metastases were produced by injected cells, three representative sections of primary tumours and metastases corresponding to the three groups of mice were analysed by immunohistochemistry against ETV5, NID1 and NUPR1 antibodies. We found expression of ETV5, NID1 and NUPR1 in HGEshC mice, whereas in HGEshNID1 and HGEshNUPR1 mice, ETV5 was expressed and NID1 and NUPR1 protein levels were reduced compared to HGEshC (Figure 35).

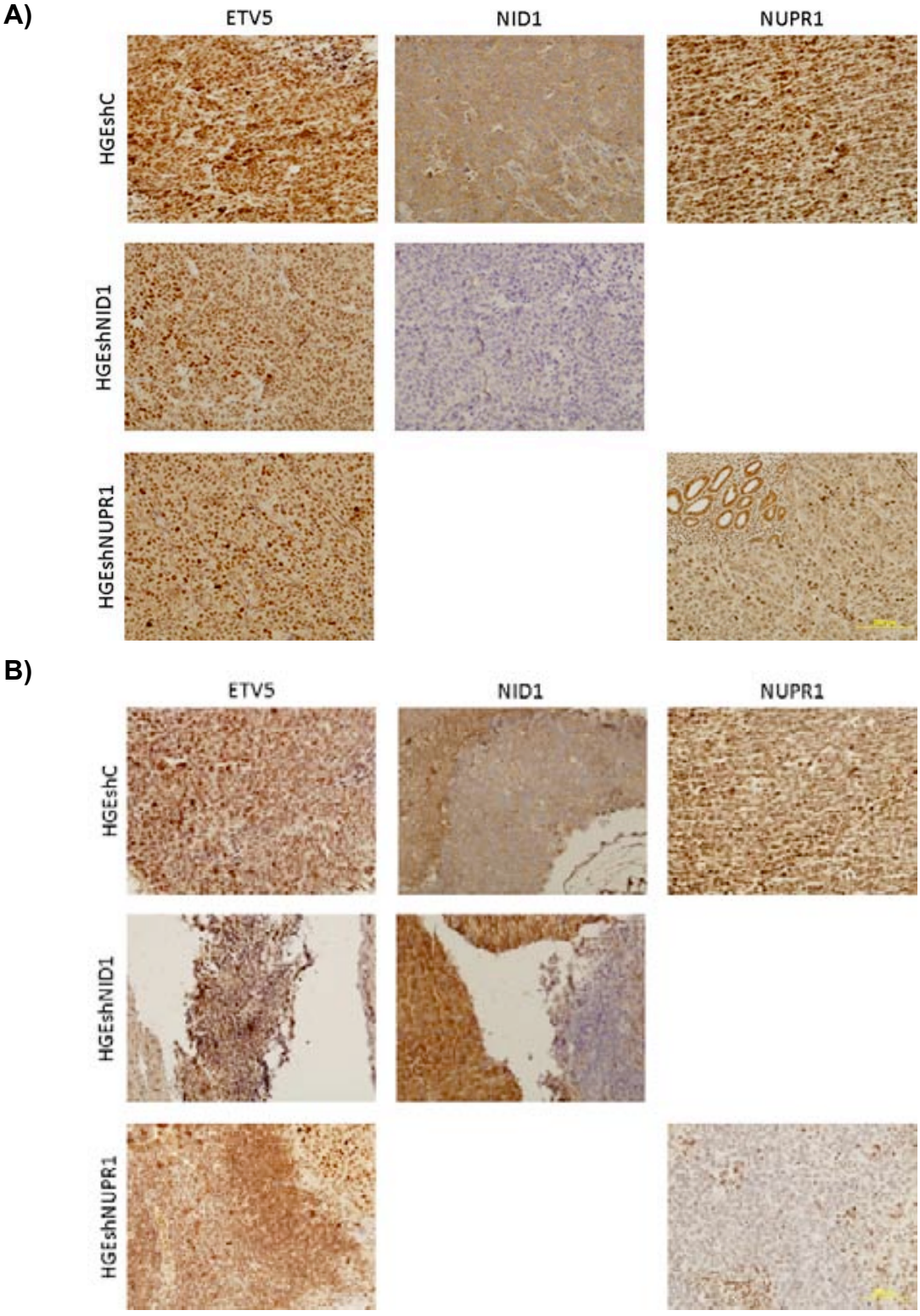

A)
ETV5
NID1
NUPR1
HGEshC
HGEshNID1
HGEshNUPR1
B)
ETV5
NID1
NUPR1
HGEshC
HGEshNID1
HGEshNUPR1

Figure 35. Three representative sections at 30X of A) primary tumours and B) metastases corresponding to the three groups of mice that were analysed by immunohistochemistry against ETV5, NID1 and NUPR1 antibodies. NUPR1 expression in HGEshNUPR1 primary tumour is shown compared to normal endometrium (upper left corner of the picture). Metastases of HGEshC and HGEshNUPR1 correspond to lymphatic metastases, while metastases of the HGEshNID1 group correspond to hepatic metastases.

The same tumour pattern was observed in the three mice groups. All tumours were infiltrating and very necrotic as a consequence of tumour growth (Figure 36). We observed that mice injected with HGEshNID1 cells presented smaller tumours than control mice. Furthermore, mice injected with HGEshNID1 and HGEshNUPR1 cells generated less metastases per mice compared to mice in the control group (*P=0.012 in the HGEshNID1 group and P=0.0474 in the HGEshNUPR1 group) (Figure 37) even though the incidence of mice with metastases was not significantly different amongst the three groups (Table 14). Altogether, these results suggest that *in vivo*, both NID1 and NUPR1 have a role in tumour growth and invasion mediated by ETV5.

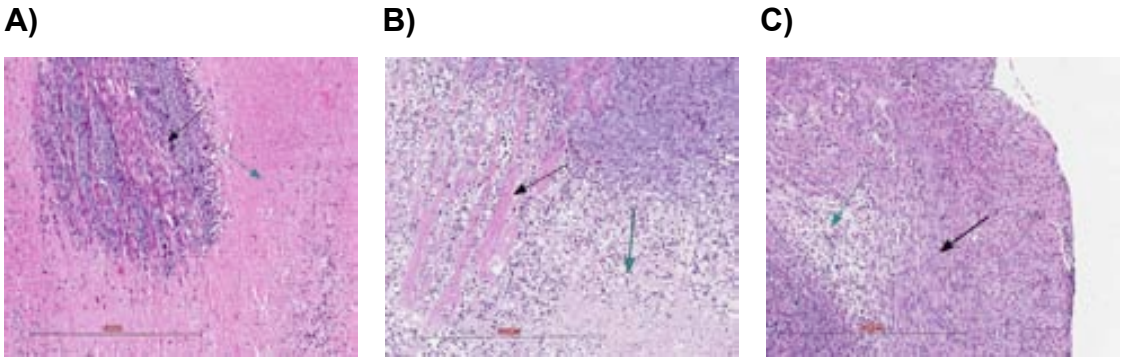


Figure 36. H&E staining of the orthotopically endometrial tumours showing the interface between the tumour and the myometrium. The tumour pattern of invasion in the three groups of mice is the same. A) HGEshC. **B)** HGEshNID1. **C)** HGEshNUPR1. The black arrow indicates the infiltrating tumour while the green arrow indicates necrosis as a consequence of tumour growth.

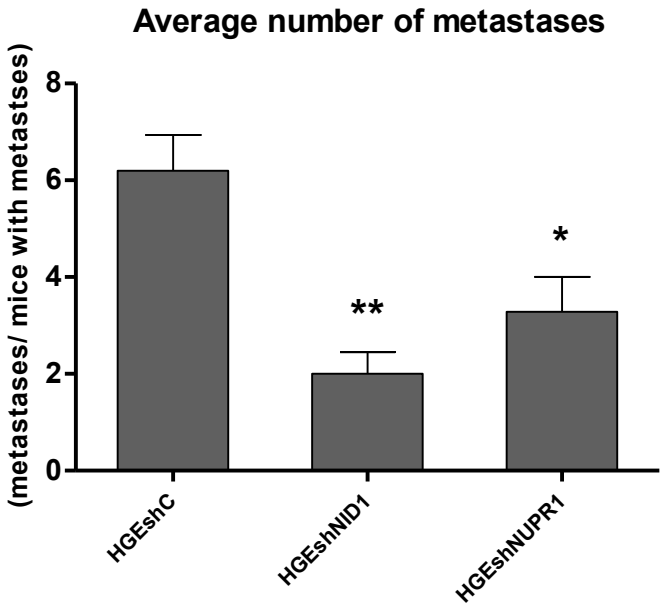


Figure 37. Mice injected with HGEshNID1 and HGEshNUPR1 cells generate less metastases. Histogram representing the average number of metastases per mice. (Mann Whitney test, **P =0.008 and *P=0.027 for HGEshNID1 and HGEshNUPR1, respectively).

In addition, we observed that in HGEshNID1 and HGEshNUPR1 mice, the number of haematogenous metastases was lower than in control mice (Chi-test P=0.05 in the HGEshNID1 group and P=0.02 in the HGEshNUPR1 group) (Table 14). The haematogenous metastases are the most aggressive, usually associated with sarcomas rather than carcinomas, which suggests that inhibition of NID1 or NUPR1 in HGE cells results in a less aggressive cell phenotype.

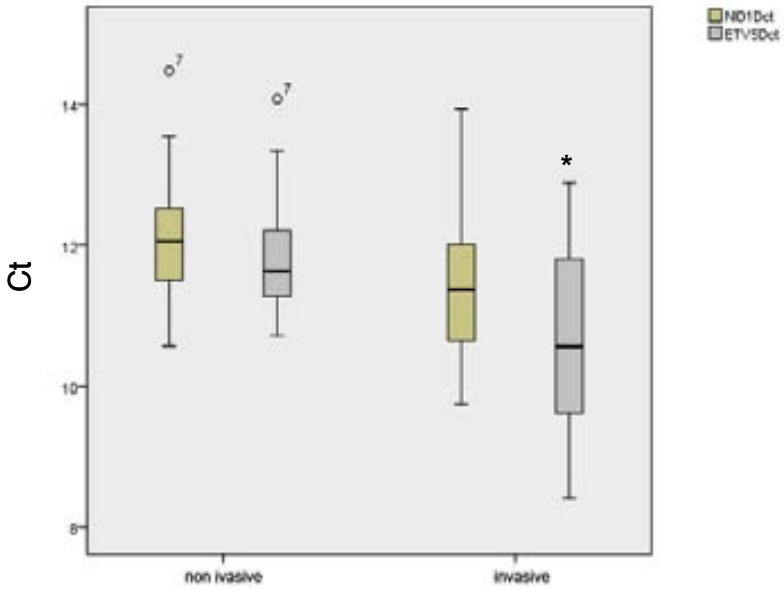
4 Expression of NID1 and NUPR1 in human endometrial tumour samples.

We have observed that ETV5 regulates the expression of the *NID1* and *NUPR1* genes through ChIP and luciferase-reporter assays (Figures 23 and 24). To verify whether an increase of ETV5 is associated with an increase in NID1 and NUPR1 in human endometrial samples, we measured by RTqPCR the mRNA expression levels of *NID1*, *NUPR1* and *ETV5* in 32 EECs corresponding to different FIGO stages and histopathological grades (Table 7). In addition, we performed IHC in a human tumour tissue microarray to measure NID1, NUPR1 and ETV5 protein expression levels.

The expression of *ETV5*, *NID1* and *NUPR1* in EC tumour samples was normalized against 4 paired control samples of 4 patients. Statistical analysis comparing the levels of expression between tumour subtypes showed a significantly higher *ETV5* (Student's T- P=0.018) mRNA expression levels in invasive tumours (FIGO stage Ib and beyond) compared with non invasive tumours (FIGO stage Ia) (Figure 38A). With regard to NID1, we found that *NID1* mRNA levels were also higher in invasive tumours compared to non invasive tumours, with a difference close to significant (Student's T, P=0.071). These data indicate that increasing levels of *NID1* (and *ETV5*) expression are associated with a more invasive phenotype. We tested whether the increase in *ETV5* mRNA expression levels was associated with the increase in *NID1* levels in the same set of human tumour samples. We found that the mRNA levels of both molecules were significantly correlated (Pearson correlation coefficient = 0.460, P=0.008) (Figure 38B), suggesting that ETV5 may contribute to the upregulation of NID1 during tumour progression.

By contrast, we could find neither a correlation between *NUPR1* and *ETV5* mRNA expression levels nor any association between *NUPR1* mRNA levels and FIGO stage or grade.

A)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B)

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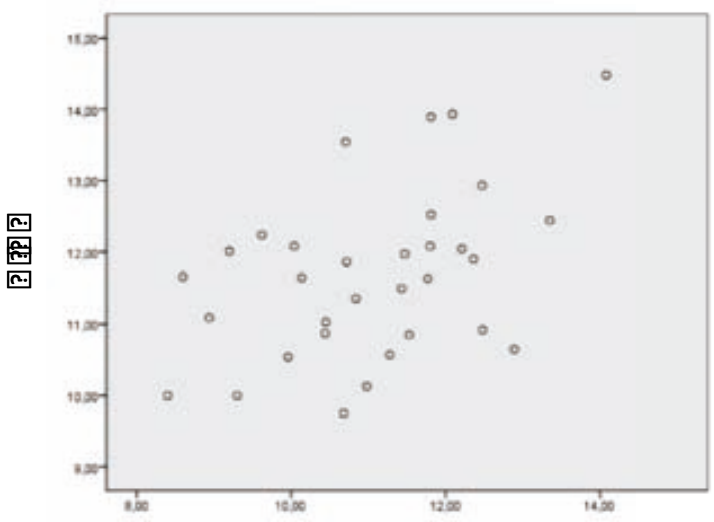
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Figure 38. *NID1* expression in endometrial tumour samples. **A)** Boxplot of *NID1* expression levels according to tumour invasiveness. A significant association between the levels of *NID1* mRNA expression and tumour invasiveness was found. **B)** The expression of *NID1* showed a significantly positive correlation with *ETV5* (Pearson's r= 0.46, P=0.008).

ETV5 has been described as specifically upregulated in the invasion front of endometrial tumours [137]. Consequently, we analyzed by RTqPCR the expression of *NID1* and *NUPR1* in 13 paired tumour samples from superficial and deep tumour corresponding to invasive ECs samples. We observed an increase in *NID1* and *NUPR1* expression in the invasion front of endometrial tumours. In the case of *NUPR1*, the increase was significant.

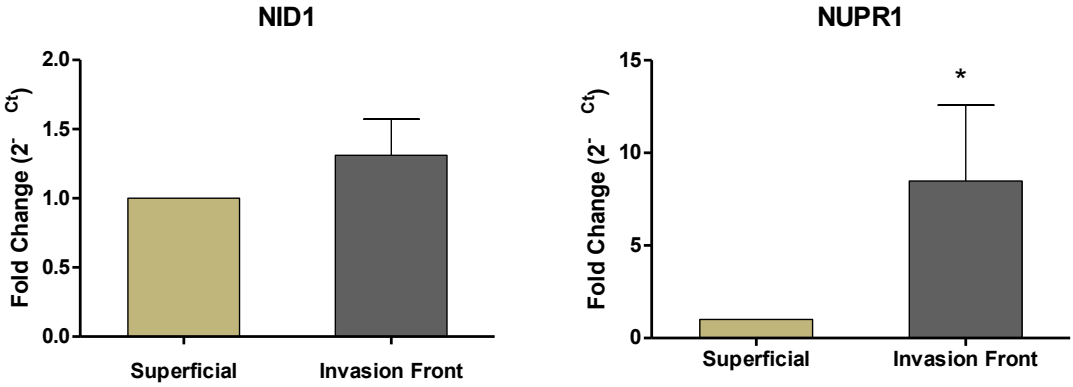


Figure 39. *NID1* and *NUPR1* are specifically upregulated in the invasion front of endometrial tumours. Relative mRNA expression of *NID1* and *NUPR1* comparing the invasive and the superficial zones of 13 paired endometrial tumour samples. GAPDH, a housekeeping gene, was used to normalize the mRNA expression of both genes.

Subsequently, IHC was performed in two different TMAs to analyze the correlation between ETV5 and NID1, and ETV5 and NUPR1 protein levels.

The first TMA, which consisted of 69 tumour samples corresponding to different histological subtypes, FIGO stages and clinical grades, was performed in the Pathology Department of the University Hospital Vall d'Hebron. The second, which consisted of 110 paired superficial and deep tissue samples from non-invasive and invasive ECs, was performed in the Pathology Department of the Hospital del Mar.

Our analyses showed a correlation between ETV5 and NID1 protein levels in EC tumour samples (Figure 40), but not between ETV5 and NUPR1. These resulats are consistent with our previous analysis on mRNA expression where we found a correlation between *NID1* and *ETV5* mRNA levels but not between *NUPR1* and *ETV5*. We also found a significant increase of NID1 and NUPR1 protein levels in the invasion front of the tumour suggesting a possible regulation of both genes by ETV5 in the myometrial infiltration process in EC (Figure 41).

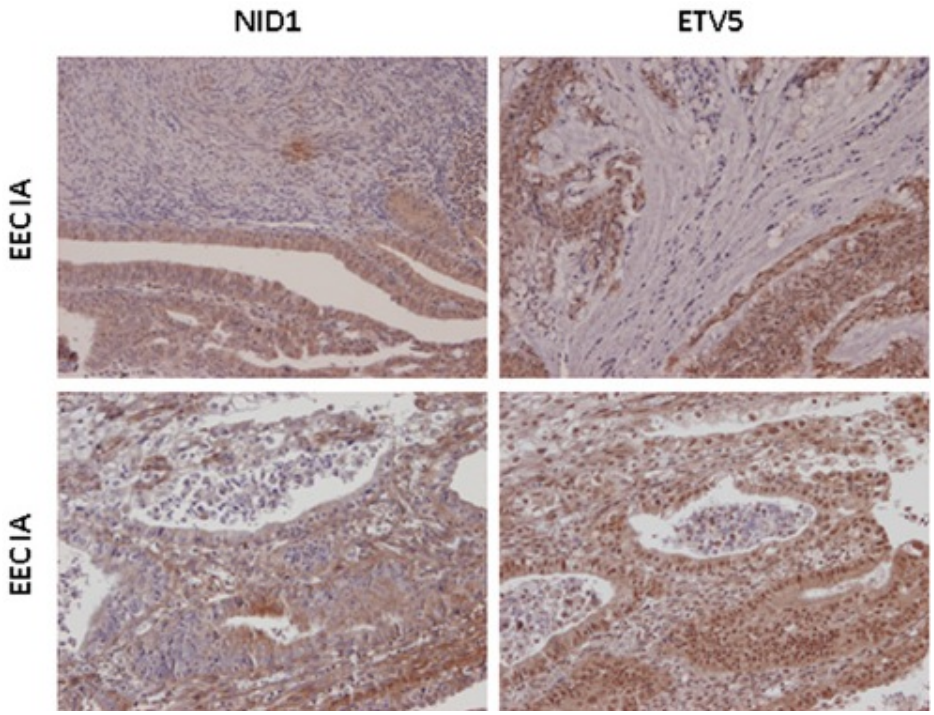
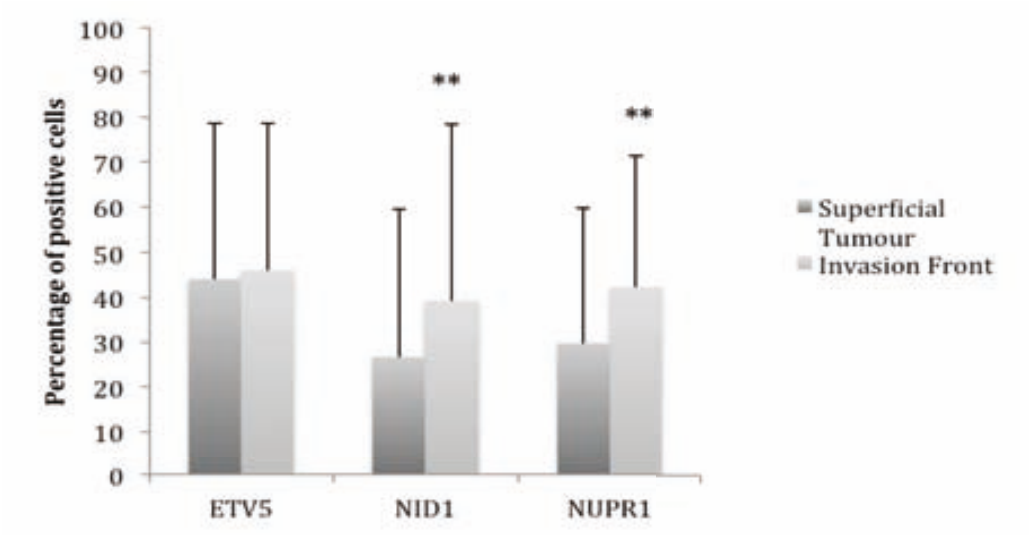


Figure 40. NID1 and ETV5 expression in endometrial tumour samples. The expression of NID1 showed a significantly positive correlation with ETV5 (Pearson's r= 0.351, P=0.01).

A)



B)

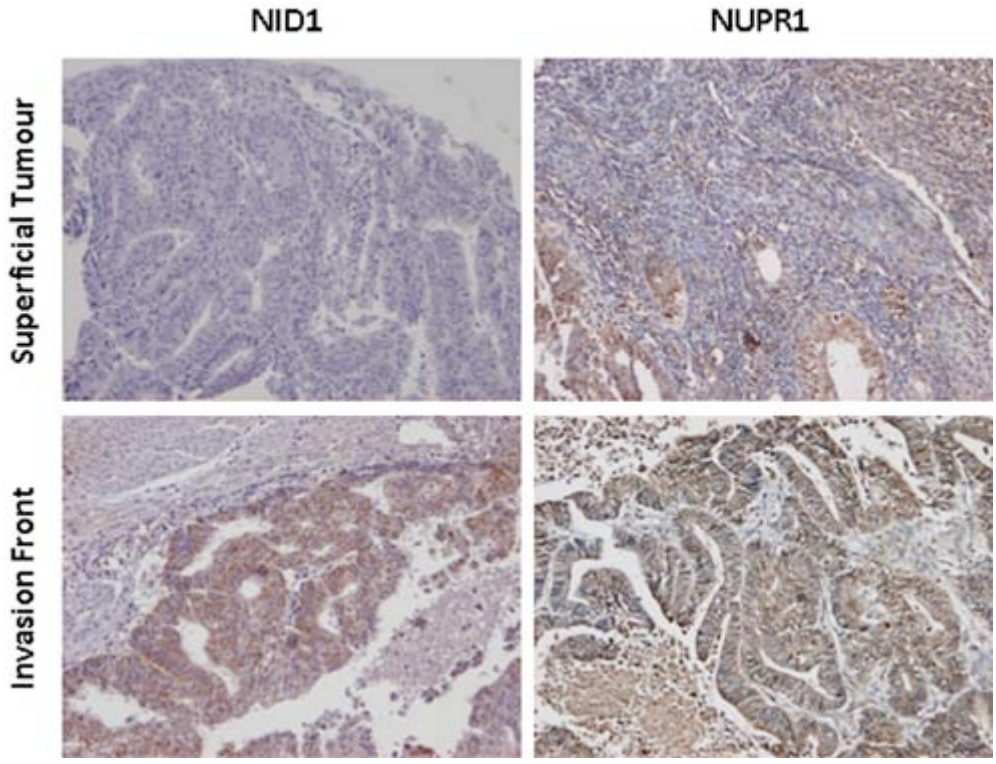


Figure 41. NID1 and NUPR1 are specifically upregulated in the invasion front of endometrial tumours. A paired t-test was used to compare the percentage of positive cells between the superficial and the invasion front of the tumor. **A)** Histogram representing the percentatge of positive cells in the superficial tumour and in the invasion front (** P= 0.01). **B)**

Representative section at 10x of the NID1 and NUPR1 upregulation in the invasion front of the tumour compared with the superficial tumour.

GLOBAL SUMMARY AND DISCUSSION

Cancer is a global disease. It affects different organs and tissues and accounts for 13% of the world's mortality. Metastasis or the spread of tumour cells to different organs is the main cause of failure in cancer treatment. Consequently, research should approach tumour invasion mechanisms to other organs to develop new therapies.

Cancer results from the accumulation of gene mutations that drive cells to an abnormal growth and tumour formation. Cancer invasion is the process by which cells break away from this primary tumour and spread through surrounding tissues. Invasion also capacitates the cells to break through blood vessels and be transported, thus enabling the establishment of secondary tumours in other locations of the body. Cancer invasion comprises several modifications in cell behaviour, in particular changes in motility and in the production of enzymes that will break down adjoining tissue. Since cancer is attributed to genetic alterations, it is indispensable to identify the genes whose changes accumulate during tumour progression. Understanding the molecular mechanisms of tumour growth and metastasis is one of the most pressing issues in cancer research. Characterization of the molecular bases of cancer invasion may improve cancer treatment via the use of therapies that target specific molecules responsible for tumour cell invasion.

Endometrial carcinoma (EC) is the most frequent infiltrating tumour of the female genital tract. Indeed, myometrial invasion implies an increase in the rate of recurrence and a decrease in survival. There are different types of endometrial cancer and the most prevalent in the developed world is endometrioid endometrial cancer (EEC). EEC has good 5-year survival rates if detected and treated at an early stage. However, when the tumour invades over a half of the myometrium, the 5-year survival rate drops dramatically, from 96% to 75% at stage Ib [86].

Endometrial carcinoma has a favourable prognosis due to the early appearance of symptoms, the most common of which are abnormal vaginal bleeding and high abdominal pain in post menopausal women [113]. However, up to 20% of patients present myometrial infiltration and/or lymph node involvement at diagnosis. As the

initial event in tumour invasion, myometrial invasion is one of the most useful prognostic factors since it increases recurrence rates after the first surgical treatment and decreases 5-year survival. New therapeutic targets to prevent cancer dissemination could be identified once the initial molecular events leading to myometrial infiltration are elucidated.

During the past few years, our group has investigated the role of myometrial infiltration in endometrial tumour progression and dissemination. In 2005, we described the upregulation of the *ETV5* transcription factor in endometrial carcinoma associated to the switch to myometrial infiltration [147]. It was consistently demonstrated that *ETV5* confers invasive capabilities to endometrial cancer cells through *MMP2* gelatinolytic activity [148]. In 2012, our group demonstrated that *ETV5* promotes EMT, resulting in endometrial tumour cells acquiring migratory and invasive capabilities [274].

This thesis aimed to further characterize the initial steps of myometrial invasion regulated by the ETV5 transcription factor in order to identify new molecules involved in myometrial invasion in endometrial carcinoma. Our approach has mainly consisted in the identification of ETV5 regulated genes involved in the regulation of cell migration and invasion.

Firstly, we analyzed by gene expression microarray technology those genes and pathways most differentially altered in Hec1A endometrial cancer cells with stable overexpression of a fusion GFP-ETV5 protein. Following research previously carried out by our group, the analysis of the molecules and connections performed by the IPA software pointed to E-cadherin and *ICAM1* as score molecules altered under the control of ETV5 (see Network 2, Figure 22, page 93). Changes in cell to cell (E-cadherin and ICAM1) and cell-matrix (integrins) adhesion contribute to the connection of mechanical and signalling pathways that govern cell migration. We have shown that the cell adhesion molecule E-cadherin is regulated by ETV5. Consequently, ETV5 overexpression is responsible for the induction of the EMT process [137]. In addition, it has been described that *ICAM1* is directly regulated by the ETV5 transcription factor [271]. Regarding Network 3, we considered *TGFβ* as

the core molecule indirectly regulating *ZEB1*, *CDH2* (N-cadherin) and *MMP2*. It has been shown that the *TGFβ* signalling pathway is modulated by *ETV5* downregulation in OV90 ovarian cancer cells [209]. Moreover, *MMP2* and *ZEB1* are also directly regulated by ETV5. Specifically, ETV5 modulates *ZEB1* expression and E-Cadherin repression leading to an epithelial to mesenchymal transition and resulting in endometrial cancer cells acquiring migratory and invasive capabilities [274]. Furthermore, ETV5 regulates *MMP2* thus conferring an increase in the invasion capabilities of endometrial tumour cells [148]. In relation to Network1, the core molecules are *NFKB* and *AKT*. NF-κB is a regulator of genes that controls cell proliferation and cell survival; many different types of human tumours have dysregulated NF-κB. Active NF-κB turns on the expression of genes that keep the cell proliferating and protect the cell from conditions that would cause its death by apoptosis. Akt regulates cellular survival and metabolism by binding and regulating many downstream effectors such as the Nuclear Factor-κB, Bcl-2 family proteins and MDM2. Akt is involved in cellular survival pathways by inhibiting apoptotic processes. Both signalling pathways have been involved in survival of tumour cells. Future work will examine if ETV5 overexpression modulates the *NFKB* and *AKT* pathways.

We selected seven significantly regulated genes to be analysed by the ChIP assay. The objective was to identify the genes that might be directly regulated by ETV5. The selection was based on statistical significance (a fold change over 1.5 and a p-value under 0.05), literature mining (genes related with cancer progression) and putative ETS binding sites in their promoter regions. ETS binding sites are small DNA consensus sequences, necessary for ETV5 binding to chromatin. The ETS domain binds DNA over a region spanning 12 to 15 base pairs, but it displays a sequence preference for only 9 bp with a central invariant 5-GGA(A/T)-3 core. The selected genes were *ANGPT-2, PLOD-2, NUPR1, EFNA5, NPR3, NID1* and *LAMP3*. Other relevant genes appear on the list. Amongst them, we find *BMPR1B, SERPINE2, FOLH1B, CDH2, FOLH1, CDK6, BIRC7* and *FN1*. They were not analysed because we could not identify either their promoter region on the 5'proximal sequence or any ETS binding sites. We found that ETV5 binds to *NID1* and *NUPR1* promoter regions by ChIP assay. Surprisingly, ETV5 did not bind to the promoter region of *ANGPT-2*.

ANGPT-2 has been described as directly regulated by the ETS-1 transcription factor [275]. It seems that differences in DNA sequence preference of most ETS proteins identified from *in vitro* studies are subtle. However, specificity in cells could derive predominantly from interactions with cofactors, including partner transcription factors. On the other hand, although several consensus binding sites for ETS proteins can be found in a wide series of promoters, only a limited number of them are actually activated by ETS transcription factors [271].

We analyzed whether ETV5 was able to induce the expression of a luciferase reporter under the control of *NID1* or *NUPR1* promoter regions. We found an increase of luciferase expression using both promoter regions, suggesting that *NID1* and *NUPR1* are transcriptionally regulated by ETV5 in endometrial carcinoma cells through the direct binding of ETV5 transcription factor to its promoter regions.

To confirm that ETV5 also regulates *NID1* and *NUPR1* in human tumour samples, we analysed the expression of the three genes in a different set of human endometrial tumour samples at mRNA level and at the protein level. We found that the expression of NID1 and ETV5 were clinically correlated in human endometrial tumour samples at mRNA level and at the protein level. Moreover, we found a significant increase of *NID1* and *ETV5* mRNA expression levels in invasive tumours (stage Ic and beyond in the old FIGO classification, which are tumours that invade more than half of the myometrium) compared to non-invasive tumours (stages Ia and Ib of the old FIGO classification). This increase in NID1 and ETV5 was not seen at the protein level. Posttranslational modifications in ETV5 and NID1 may explain this result. NID1 is secreted to the extracellular matrix while ETV5 is modified posttranslational by several mechanisms such as ubiquinitation to regulate its function as a transcription factor. Although we did not observe a correlation between the expression of NUPR1 and ETV5 at mRNA level or at the protein level, or an increase in NUPR1 expression in invasive tumours compared to non invasive tumours, we observed an increase of NUPR1 and ETV5 mRNA expression in the invasion front of the tumour (see discussion below). These results suggest that ETV5 may contribute to the regulation of *NID1* and probably of *NUPR1* during endometrial tumour progression and invasion.

In contrast with the superficial zone, the area of the tumour inside the uterus where no active invasion is observed, our group reported an increase of ETV5 expression in the invasion front of the tumour, the area in close contact with the myometrium and where tumour cells are actively invading the surrounding stroma. In agreement with these results, we observed an overexpression of *NID1* and *NUPR1* in the invasion front of the tumour at mRNA level and at the protein level, compared to their paired superficial zone.

Heterogeneity among different areas of the tumour has been abundantly described. It has been observed that gene and protein expression are dynamically adapted in the invasive front of the tumour, as shown by changes associated with the phenotype of the epithelial cells that become invasive, i.e., epithelial to mesenchymal transition, the release of growth factors or induction of metabolic stress, as well as by tumour modifications induced by the microenvironment, for instance with the cytokines or inflammatory mediators that enhance tumour growth and invasion [276-279]. We suggest that NID1 and NUPR1 are actively cooperating with ETV5 in the acquisition of an invasive phenotype to jointly drive cancer invasion.

In conclusion, ETV5 regulates *NID1* and *NUPR1* expression in endometrial carcinoma. More specifically, an upregulation in the invasion front of the tumour at mRNA level and at protein level for both genes has been found.

In order to study the ETV5 function mediated by *NID1* and *NUPR1* involved in endometrial cancer invasion, we downregulated both genes in GFP-ETV5 overexpressed Hec1A cell lines. We performed different functional studies of HGE cells with NID1 or NUPR1 inhibition to assess proliferation, adhesion, apoptosis, migration and invasion capacities using *in vitro* assays. The proliferation assays of the cell lines did not show any difference between them. Instead, using the migration transwell assay and videomicroscopy to follow the cells, we found a decreased migration capability of the ETV5 overexpressed cell line with NUPR1 inhibition. Similar results have been reported by Sandi et al., who described that NUPR1 expression controls pancreatic cancer cell migration. Indeed, knocking-down NUPR1 in pancreatic cancer cells *in vitro* decreased migration and invasion and increased cell

adhesion. Overexpression of NUPR1 produced the opposite effect [280]. Unlike Sandi et al., we could not find a decrease in cell invasion in HGE cells with downregulation of NUPR1, which suggests that in these cells invasion is mediated by other molecules and NUPR1 has not a prevalent role. NUPR1 belongs to the HMG of transcriptional regulators [242], which controls the expression of several target genes by regulating chromatin accessibility. Overexpression of NUPR1 has been found in pancreatic, pituitary, breast and thyroid cancer [281]. Some reports indicate that NUPR1 expression is a pivotal mediator of tumorigenesis *in vivo* [295]. Opposing effects of NUPR1 depending on the physiological scenario have been described. For example, NUPR1 promotes tumour growth and aggressiveness and protects tumour cells from apoptosis; it can simultaneously act as a tumour suppressor [282]. NUPR1 is also overexpressed in breast tumours, establishing primary carcinomas and mediating the growth-promoting function in early development but not in later stages of tumorigenesis or metastasis [283].

Our group had described ETV5 as a protective factor of apoptosis under oxidative stress conditions, partly through direct regulation of the Hep27 protein. It has been shown that Hec1A cells with ETV5 overexpression generate oxidative stress as a consequence of increased migration and invasion [273]. In order to check whether NUPR1 had a protective role against apoptosis induced by oxidative stress in HGE cells, we measured apoptosis induction in HGEshNUPR1 cells and controls, quantified by a caspase activity assay, in basal conditions and after treatment with H_2O_2. Unexpectedly, Hec1A cells with ETV5 overexpression and NUPR1 inhibition present a decrease of apoptotic capacity under stress conditions. Although in most cell types an increased intracellular level of NUPR1 appears to be associated with protection against apoptosis, NUPR1 function during apoptosis is complex and ambiguous because it can vary depending on the cell's biological context. NUPR1 interacts with prothymosin-α, which inhibits mitochondria-dependent apoptosis. In other situations, the expression of NUPR1 activates the *ATF4*, *CHOP* and *TRB3* genes to transduce the ER-stress-associated signal. The activation of the NUPR1/TRB3 pathways inhibits mTOR and Akt activities and induces stress-associated apoptosis as a consequence [284]. Commonly, cancer cells activate the ER-stress response after luminal accumulation of misfolding proteins, hypoxia or starvation. Our results

suggest that under high oxidative stress conditions induced by the addition of H_2O_2, the expression of *NUPR1* induced by ETV5 leads to the activation of the ER-stress response and cell death.

Regarding NID1 function, the inverted invasion transwell assay showed a decrease of invasion of cells with ETV5 overexpression and NID1 inhibition. Cell invasion is a multistep process which requires adhesion, proteolysis of extracellular matrix components and migration. Laminin is the major partner of NID1 in the extracellular matrix. Moreover, we have previously reported that overexpression of ETV5 in Hec1A cells increases cell adhesion to laminin and collagen I. We next checked the adhesion properties of HGEshNID1 cells to laminin and collagen I compared to HGEshC control cells. We found a significant decrease in the adhesion to laminin in cells with ETV5 overexpression and NID1 inhibition, which suggests that the increase in cell adhesion to laminin of HGE cells is mediated by NID1. In contrast, cell migration was not reduced in HGE cells with downregulation of NID1, suggesting that NID1 mainly contributes to the increase in cell adhesion and invasion but not cell migration. In fact, it has been shown that NID1 interacts with the β1 family of integrin receptors and with αvβ3 integrin modulating cell adhesion, but not cell migration [265]. Moreover, NID1 has been described as a candidate ligand for tumour endothelial marker 7 during angiogenesis [263]. Recent studies have demonstrated that ECM proteins play important roles within the tumour microenvironment. For example, proteoglycans help drive multiple oncogenic pathways in tumour cells and promote critical tumour-microenvironment interactions in brain cancer [285]. The ECM proteins initiate downstream signalling pathways that lead to proliferation, invasion, matrix remodelling and dissemination of premetastatic niches in other organs. Mueller and Fusenig in 2004 and Kalluri and Zeisberg in 2006 demonstrated that alterations in tumour suppressor genes and oncogenes during tumour progression cause cancer cells to activate adjacent stromal components and induce the release of cytokines, growth factors and ECM proteins into the tumour stroma to create a microenvironment conducive to growth and dissemination [286, 287]. In addition, deposition of ECM proteins such as collagen I and IV, fibronectin, laminin and matricellular proteins leads to matrix remodelling and the subsequent release of proteases such as

metalloproteinases (MMPs) and cathepsins, which degrade the basement membrane and initiate a proinvasion programme for tumour cells [288].

Cell migration and invasion are two necessary mechanisms of tumour progression and dissemination. To migrate, cells must modify its shape and firmness; firstly, they interact with adjoining tissue structures through the growth of cell protrusions that originate from the modulation of the actin cytoskeleton and next, they generate focal contact formation by means of cell adhesion to interact with the extracellular matrix. To invade, different types of proteases must degrade the extracellular matrix. In epithelial cancers such as endometrial carcinoma, migration and invasion mechanisms act usually together [289]. Our results suggest that NUPR1 and NID1 may act cooperatively with regard to modulation of cell migration, cell adhesion and cell invasion, thus contributing to cancer progression and dissemination induced by ETV5 upregulation.

To understand the mechanism underlying the effect of NID1 and NUPR1 in cell invasion and migration, we analysed the expression of EMT markers in HGEshNID1 and HGEshNUPR1 cells. We have previously shown that overexpression of ETV5 in Hec1A cells induces the expression of the *ZEB1* repressor, the loss of E-cadherin and the induction of N-cadherin expression, events associated to the EMT programme. These changes coincide with an increase in cell migration and invasion in Hec1A cells overexpressing ETV5 [137]. Interestingly, we observed that the increase in the expression of N-cadherin in HGE cells was reverted in HGEshNID1 and HGEshNUPR1 cells. In contrast, the expression of E-cadherin was maintained in the four ETV5 overexpressed cell lines.

Usually, the increase of the N-cadherin expression correlates with a downregulation of E-cadherin expression and an increase of cell motility and invasion capabilities. However, in some tumour cell lines N-cadherin expression is an event dominant over E-cadherin and cells increase their migratory and invasive capacity regardless of E-cadherin expression [290]. The reduction of N-cadherin expression may explain the reduction in cell migration and invasion of HGEshNID1 and HGEshNUPR1 cells. There are many reports on N-cadherin expression in relation with the migration

capability of the tumour cells. Recently, Xu et al. reported that N-cadherin inhibits osteogenesis but promotes migration of bone marrow-derived mesenchymal stem cells [291]. Moreover, Shih et al. described that the N-cadherin-mediated cell-cell adhesion supports cell interactions between migrating cells in a more physiologically relevant 3D matrix [292].

Our *in vitro* results were further validated using an orthotopic endometrial cancer mouse model [267]. This mouse model had been previously established in our lab [267] and showed a high generation of tumour and metastases. The dissemination pattern imitates the infiltrative process and metastatic behaviour of endometrial cancer. In our experiment, endometrial cancer cells were injected transmyometrally in the mice uteri and let to grow for approximately 8 weeks. At this point, mice were sacrificed. We observed that the tumours with NID1 inhibition were smaller. Likewise, we observed that the average number of metastases decreased when NID1 and NUPR1 were inhibited. Similar results were obtained by Vasseur et al., who injected subcutaneously or intraperitoneally fibroblast cells with overexpression of NUPR1 and with NUPR1 silenced. They observed that mice injected with NUPR1 overexpression cells produced tumours, whereas the mice injected with silenced NUPR1 fibroblasts did not [221]. Research carried out by Sandi et al. showed that xenograft pancreatic tumour development is dependent on NUPR1 expression; Panc-1 cells transfected with siNUPR1 did not produce tumours in athymic nude mice, whereas NUPR1-overexpressed-Panc-1 cells did [280]. On the other hand, there are no reports in the literature describing a role of NID1 function in cancer development *in vivo*.

In summary, our results suggest that the ETV5 transcription factor regulates *NID1* and *NUPR1* transcriptionally. *NID1* regulation by ETV5 enhances cell invasion and cell adhesion to the extracellular matrix, and *NUPR1* regulation by ETV5 enhances cell migration in endometrial carcinoma. We propose that NID1 and NUPR1 regulation of cell migration and invasion may be in part mediated by the regulation of N-cadherin. The increase in ETV5, NID1 and NUPR1 expression is preferentially at the invasion front of the tumour, where tumour cells infiltrate the myometrium. NUPR1 has been abundantly described in tumour progression and dissemination, whereas NID1 has not

been yet associated with cancer progression. To our knowledge, this is the first study that describes a role of NID1 and NUPR1 in endometrial carcinogenesis.

From a clinical point of view, tumour invasion defines the frontier between tissue-restricted carcinoma and disseminated tumour cells. The former is associated with a good outcome since surgery, the best current therapeutic option, is highly successful in eliminating the disease. The latter is associated with poor prognosis and a dramatic decrease in survival; radiotherapy and chemotherapy are the treatment of choice, although they show limited efficacy. Consequently, understanding the molecular events related to myometrial infiltration and distant metastases becomes an essential requirement for the design of new therapeutic approaches against the most aggressive types of endometrial cancer. We propose NID1 as a potential clinical target to reduce endometrial tumour growth and tumour metastases. NID1 has the advantage to be an extracellular protein, i.e., it is more accessible than an intracellular molecule. NID1 function could be blocked by an antibody against the domains involved in adhesion to the extracellular matrix. Our group plans to develop further experiments to address this point, in particular looking for antibodies that block NID1 cell adhesion and invasion *in vitro* and *in vivo*.

In conclusion, the data presented in this thesis contributes to the elucidation of the molecular mechanisms involved in endometrial cancer dissemination. Understanding the molecular basis of myometrial invasion in endometrial cancer will contribute to the development of more specific and more effective therapeutic strategies.

CONCLUSIONS

Our conlcusions are:

1. ETV5 regulates NID1 and NUPR1 mRNA and protein expression in Hec1A endometrial cancer cells by binding to their promoter regions and regulating their transcription.
2. The inhibition of NID1 in Hec1A cells with GFP-ETV5 overexpression reduces cell invasion and cell adhesion to laminin but does not modulate cell proliferation or cell migration *in vitro*.
3. The inhibition of NID1 in Hec1A cells with GFP-ETV5 overexpression decreases tumour growth and tumour metastases *in vivo*.
4. The inhibition of NUPR1 in Hec1A cells with GFP-ETV5 overexpression reduces cell migration and cell apoptosis in oxidative stress conditions *in vitro* but does not modulate cell proliferation or cell invasion.
5. The inhibition of NUPR1 in Hec1A cells with GFP-ETV5 overexpression decreases tumour metastases but not tumour growth *in vivo.*
6. The expression of *NID1* and *ETV5* correlates significantly in human endometrial carcinoma samples at mRNA level.
7. The expression of *NID1*, *NUPR1* and *ETV5* is upregulated in the invasion front of the EEC human samples at mRNA level.
8. We propose that NID1 and NUPR1 are two target genes of ETV5 transcription factor involved in endometrial cancer initiation and progression.
9. NID1 is a new potential clinical target to reduce endometrial tumour growth and invasion.

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