# INITIATION AND PROGRESSION OF MÜLLERIAN DUCT DERIVED MALIGNANCIES

Paul van der Horst

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**Initiation and progression of Müllerian duct derived malignancies**
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# INITIATION AND PROGRESSION OF MÜLLERIAN DUCT DERIVED MALIGNANCIES

## Ontstaan en progressie van maligniteiten van de Müllerse gang.

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# Contents

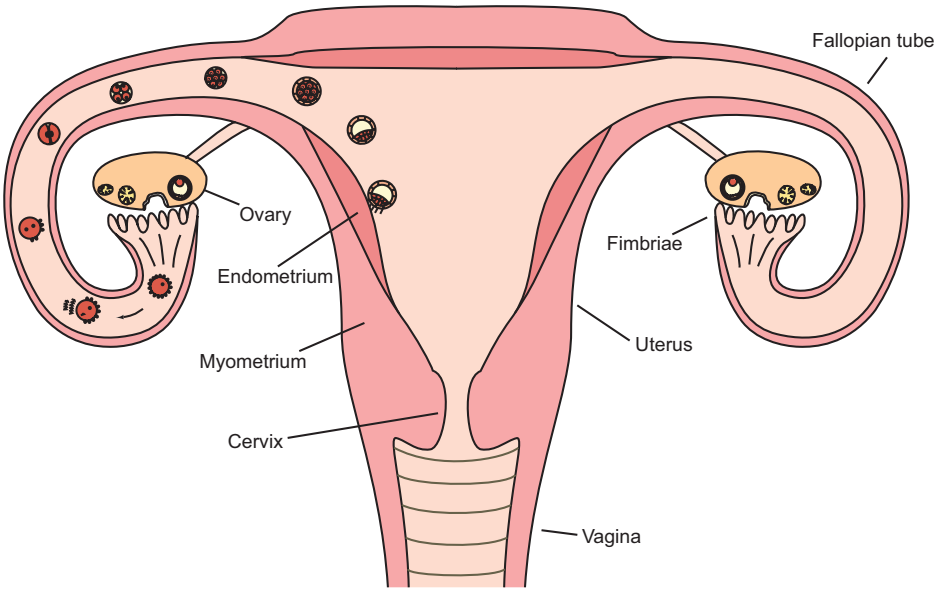
# Chapter 1

## General introduction

## The female reproductive system

The female reproductive system consists of the internal and external genitalia. The external genitalia are formed by the vulva, which includes the clitoris, labia majora and minora, urethral orifice and vestibule of the vagina (lower part of the vagina). The internal genital system is located within the pelvis and can be divided into the reproductive tract and the two ovaries (Fig. 1). The reproductive tract consists of the Müllerian duct-derived upper vagina, uterus and two fallopian tubes (oviducts) and functions to transport and guide semen to the oocyte in order to fertilize it (vagina, uterus, fallopian tubes), to hold and nurture the fertilized oocyte during its completion of development from embryonic to fetal stage (uterus) and to form the birth canal (uterus, vagina). The ovaries produce oocytes and secrete hormones necessary for secondary sexual development, regulation of the menstrual cycle, facilitation of implantation and maintenance of the early pregnancy.

**Figure 1: An overview of the internal reproductive organs.**




### Embryonic development of the reproductive system

Determination of gender starts at fertilization, when a paternal Y (male determination) or X (female determination) chromosome joins the maternal X chromosome in the oocyte. Even though gender is determined during these first moments of pregnancy, females and males are indistinguishable in the first six weeks of development: the indifferent stage. True phenotypic differentiation of gender does not start until the seventh week of pregnancy with differentiation of the gonads, followed by differentiation of the sexual duct system and finally differentiation of the external genitalia and secondary sexual characteristics (such as breast development, hair patterning and body configuration)[1-3].

*Development of the ovaries*

Gonadal development starts in the caudal part of the ventromedial border of the mesonephros when gonadal rigdes become prominent in the coelomic cavity during the fifth week of pregnancy. These early gonads develop from migrating somatic cells, derived from the mesonephros, the surrounding mesenchymal and coelomic epithelium, and primordial germ cells migrating from the endodermal layer on the posterior wall of the yolk sac along the mesentery of the hindgut into the gonad[1, 2]. As described earlier, until the seventh week of pregnancy the gonads are indifferent. The initial development of the gonads into either a male or female phenotype, however, is depended on the presence of the *SRY* gene, located on the male Y-chromosome[3]. Under the influence of *SRY, SOX9* is expressed and *DAX1* is inhibited, which leads to the formation and final differentiation of Sertoli cells and eventually gonadal development into testis. In absence of *SRY*, *DAX1* is continuously expressed, causing suppression of testis formation and development of the gonads into ovaries[4]. The presence of viable primordial germ cells is crucial for ovarian differentiation and if primordial germ cells fail to reach the primitive gonads or if they are abnormal, the gonads regress resulting in streak (vestigal) ovaries[2]. Upon entry into the ovary, primordial germ cells nest in the secondary sex chord, concentrated in the cortical region of the ovary, and are now called oogonia. While most oogonia continue to proliferate by mitosis, some oogonia in the inner medulla enter the prophase of the first meitotic division upon which they are called oocytes. These oocytes become surrounded with granulosa cells and form primordial follicles. Meiosis of these oocytes proceeds until the diplotene stage of the prophase of the first meitotic division and at that point is arrested until the blockade is removed during reproductive life[1, 2].

*Development of the reproductive tract*

The reproductive tract, consisting of the upper vagina, uterus and fallopian tubes, stems from the embryonic paramesonephic or Müllerian duct. During the sixth week of pregnancy, the Müllerian duct develops from a specific subset of cells in the anterior region of the coelomic epithelium adjacent to the mesonephros. Müllerian duct initiation is dependent on WNT signaling and under the influence of WNT4 secreted by the coelomic epithelium, *LIM1* and *PAX2* expressing mesoepithelial cells invaginate, thereby creating a coelomic opening[5-7]. Upon invagination, the primitive Müllerian duct extends and under the influence of WNT9B secreted by epithelial cells of the Wolffian duct, posterior elongation is initiated and the Müllerian duct extends further towards the cloaca[8]. Final outgrowth of the Müllerian duct is completed by widespread proliferation along the developing duct and at the growing tip and as a last step, both Müllerian ducts fuse to form the uterovaginal tube, which is completed at 16 weeks[5, 9].

During the indifferent stage, both the Wolffian and the Müllerian ducts are present. If the gonads develop into testes, testosterone secreted by the testicular Leydig cells and anti-Müllerian hormone (AMH) secreted by testicular Sertoli cells, cause the Wolffian ducts to further differentiate in the male reproductive tract and causes the Müllerian ducts to regress. However, if the gonads develop into ovaries or if gonads are absent, testosterone and AMH are not secreted, and therefore the Wolffian ducts regress and the Müllerian ducts further differentiate[2].

Differentiation of the primitive Müllerian duct into the components of the reproductive tract, the upper two third of the vagina, uterus and fallopian tubes, is dependent on WNT7A expressed by oviductal and uterine epithelial cells and WNT5A expressed by uterine, cervical and vaginal mesenchymal cells[10, 11]. Next to WNT signaling, differentiation of the Müllerian duct is further mediated by spatially restricted members of the HOX family of homeobox genes. *HOXA9* is expressed in the developing tubal epithelium, *HOXA10* in the developing uterus, *HOXA11* in the lower uterine segment and cervix and *HOXA13* in the upper two third of the vagina[12]. The lower one third of the vagina is formed by epithelial cells from the urogenital sinus under the influence of the Wolffian duct[1]. This process, however, is still poorly understood.

*Development of the external genitalia*

Similar to the gonads and reproductive tract, the external genitalia are indifferent during their first development. The indifferent external genitalia are derived from mesodermal tissue near the cloaca and in the fourth week of pregnancy the genital tubercle develops ventral from the cloaca, flanked by a pair of genital folds and genital swellings. In the center of the genital folds, the urogenital sinus opens into the abdomen. Under the influence of dihydrotestosterone, the genital tubercle elongates and forms the penis, the urogenital folds fuse and enclose the urethra and the genital swellings enlarge and fuse to form the scrotum. However, if testes are absent, dihydrotestosterone is not synthesized and the indifferent external genitalia differentiate into a female phenotype. Here, the genital tubercle inverts and becomes the clitoris, the genital folds develop into the labia minora, the genital swellings become the labia majora and the urogenital sinus forms the upper vagina and the vestibule in which the urethra and vagina open[1, 2, 13].

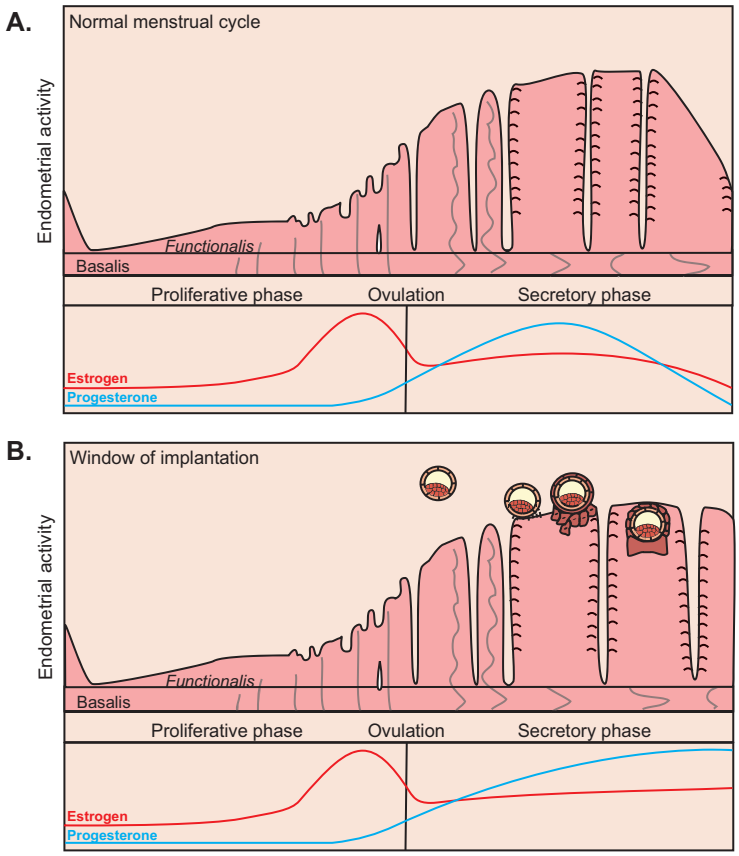
## The menstrual cycle:

The menstrual cycle involves cyclic changes in the ovary and the uterus. The ovarian cycle includes the follicular phase, ovulation and the luteal phase. The endometrial cycle includes the menses, proliferative phase and the secretory phase. The reproductive phase of life starts at the menarche, which marks the menses of the first menstrual cycle usually around 13 years of age, and continues until approximately 50 years of age. The menstrual cycle is the effect of the ovary secreting hormones during production of oocytes for fertilization. Under the control of estrogen and progesterone the reproductive system undergoes functional and structural changes to optimize uterine conditions for embryo implantation and subsequent placentation (Fig. 2a-b).

The uterus can be divided in two functional layers: the outer myometrium and the inner endometrium. The endometrium facilitates implantation, development and outgrowth of the embryo and can be divided into two layers: the functionalis and basalis. Every month, the functionalis is shedded during menstruation, which marks the start of a new menstrual cycle (Fig. 2a). During the first two weeks of the menstrual cycle, the proliferative phase, estrogens produced in the ovary induce proliferation of the endometrium and thereby generate a new functionalis. In the ovary, this first phase of the menstrual cycle is called the follicular phase, during which the

follicle matures and prepares to release its oocyt for fertilization. As stated, during this phase the ovary produces estrogens crucial for endometrial proliferation (Fig. 2a). However, the cells present in the ovary are not capable to synthesize estrogens in one step and therefore collaboration between theca and granulosa cells is vital for estrogen production. Under the influence of the pituitary secreted luteinizing hormone (LH), thecal cells convert cholesterol into androstenedione, using 17α-hydroxylase, which serves as a precursor for estrogen. Upon diffusion through the basal membrane into surrounding granulosa cells, androstenedione is then converted into estrogen (estradiol) by aromatase and 17β-HSD under the influence of the pituitary secreted follicle-stimulating hormone (FSH). Pituitary secretion of LH and FHS, in its turn, is under control of GnRH secreted by the hypothalamus and inhibin, activin and estrogen secreted by the ovary. In addition to the estrogenic effect on the endometrium, estrogens also influence the cervix by stimulation of cervical mucus production, which allows the spermatozoa easy excess to the uterine cavity.

**Figure 2: The endometrial cycle.**




(A + B) Functional and structural changes of the endometrium under the control of estrogen and progesterone during the normal menstrual cycle (A) and the window of implantation (B). Figure adapted from Vd Horst et al. (2012) Mol Cell Endocrinol. 358(2):176-184.

After ovulation, during which the oocyt is released from the ovarian follicle into the fallopian tube, the second half of the menstrual cycle or secretory phase starts (Fig. 2a). During this phase the endometrium prepares for implantation of the fertilized ovum. Here, progesterone, counterbalances the proliferative effects of estrogen and is responsible for the induction of differentiation of the endometrium necessary for optimal implantation. The corresponding ovarian phase is called the luteal phase, during which, progesterone is synthesized by LH stimulated ovarian conversion of cholesterol in the corpus luteum. In contrast to estrogen production, progesterone is not synthesized by both thecal and granulosa cells, but by luteinized granulosa cells of the follicle alone.

Progesterone-induced endometrial differentiation is characterized by induction of secretory activity of the glands, attraction of natural killer cells and transformation of endometrial stromal cells into decidual cells, a process called decidualization. Furthermore, progesterone inhibits passage of spermatozoa through the cervix by induction of very thick and acidic mucus production. If fertilization is absent, progesterone production declines, the functional layer of the endometrium degenerates and the menstrual cycle restarts at menses. In case a zygote is formed (Fig. 2b), embryonic surface cells, called trophoblastic cells, will produce human chorionic gonadotropin (HCG), stimulating the corpus luteum to continue the secretion of progesterone which inhibits shedding of the functionalis layer of the endometrium (Fig. 2a)[2, 13-17].

## The role of WNT/β-catenin signaling during the menstrual cycle

The WNT signalling pathway has been shown to be a key regulator in development and disease since the discovery of *Wnt1* in 1982[18, 19]. In humans, 18 WNT proteins have been identified and upon binding of these WNT proteins to their Frizzled receptor the WNT/β-catenin signalling pathway can be activated[19, 20]. Central to canonical WNT/β-catenin signalling is the degradation complex, which consists of the scaffold proteins AXIN1 and AXIN2 (conductin), β-catenin, APC (adenomatosis polyposis coli), CK1 (casein kinase I) and GSK3β (glycogen synthase kinase 3 beta). In absence of WNT, β-catenin is phosphorylated by GSK3β and CK1, leading to its degradation. However, upon binding of WNT, the Frizzled receptor cooperates with a member of the LRP family and as a result, the degradation complex is dissociated and β-catenin becomes stably available in the cytoplasm[21, 22]. Stabilized β-catenin can now translocate to the nucleus where it displaces the transcription repressor Groucho (TLE), which leads to TCF/LEF transcription factor family regulated WNT target gene transcription[23].

WNT/β-catenin signaling is thought to be implicated in regulation of the regular menstrual cycle, a process extensively described in chapter 2 of this thesis. During the proliferative phase of the menstrual cycle increased estrogen levels stimulate WNT/β-catenin signaling in order to enhance proliferation, while in the secretory phase, progesterone levels inhibit WNT/β-catenin signaling thereby counterbalancing estradiol-induced proliferation and enhancing differentiation. This was confirmed by the fact that nuclear β-catenin staining is observed during the proliferative phase of the menstrual cycle, while nuclear β-catenin is absent during the second half of the menstrual

cycle[24]. Furthermore, exogenous administration of estrogen resulted in accumulation of nuclear β-catenin in endometrial cells and upon viral-induction of the WNT/β-catenin inhibitor *SFRP2,* estrogen induced proliferation was inhibited[25]. The relationship between the menstrual cycle was further confirmed using gene expression profiling, where WNT/β-catenin signaling activating factors, such as *WNT4, WNT5A, WNT6* and *WNT7A* were found to be upregulated in the proliferative phase, in contrast to WNT/β-catenin signaling inhibitors, such as *DKK1* and *FOXO1*, which were upregulated during the secretory phase[26-28]. In addition, using data obtained from hormone treated postmenopausal women it was shown that many targets and components of the WNT signaling pathway were regulated by estrogen and progesterone[28-30].

## Endometrial cancer

Worldwide, more than 288.000 women are diagnosed with endometrial cancer each year, making it the most common gynecological malignancy and the fourth most common female malignancy in developed countries[31]. In the Netherlands, in 2008, more than 1800 women were diagnosed with endometrial cancer, accounting for an incidence of 22,4 per 100.000 women and a cumulative risk of endometrial cancer up to 75 years of age of 1,55%[31]. Unfortunately, due to the increase in life expectancy and a rising presence of endometrial cancer risk factors within the world population, a substantial increase in endometrial cancer incidence is expected in the near future[32].

### Risk factors

Age is the most important risk factor for endometrial cancer as approximately seventy-five percent of all cases occur in postmenopausal women[33]. Furthermore, obesity was found to be a major risk factor due to its associated high estrogen level caused by conversion of androgen into estrogens within the fat tissue[34, 35]. Next to age and obesity, other important risk factors for endometrial cancer related to prolonged exposure to high levels of estrogens include long-term exposure to estrogen therapy, polycystic ovary syndrome (PCOS), early menarche, late menopause and null parity[33, 36-38]. Additional risk factors are long-term use of Tamoxifen, endometrial cancer family history in the first degree, *BRCA1* mutation and HNPCC family (Lynch) syndrome[39-43]. In contrast, factors decreasing long term unopposed estrogen levels such as smoking, oral-contraceptive use, grand multi parity and a diet with phytoestrogens, decrease the risk of endometrial cancer[44-47].

### Symptoms and diagnosis

The most prominent and early symptom of endometrial cancer is abnormal uterine bleeding or spotting. Even though uterine bleeding is associated with many other diseases, all postmenopausal women with uterine bleeding should be assessed for endometrial cancer. Additional symptoms include nonspecific symptoms such as lower abdominal pain or pelvic cramps. Transvaginal ultrasonography (TVU) is the first step in diagnosis and is used to assess the endometrial thickness and irregularity of the endometrial-myometrial border. Final diagnosis of endometrial cancer is done histologically using endometrial tissue obtained by Pipelle biopsy or hysteroscopy[33, 48].

## Pathology

In case of endometrial cancer, using histological assessment of the endometrial biopsy, endometrioid adenocarcinoma is identified in 80% of cases[33]. Other subtypes of endometrial cancer are mucinous, serous, clear-cell, mixed Müllerian, squamous-cell, transitional cell, small-cell and undifferentiated carcinoma[49]. Like many other types of cancer, endometrial carcinoma can be further divided into two subgroups based on their differentiation. Most endometrial cancers are well to moderately differentiated and are known as type I endometrial cancer. Type I endometrial carcinomas are mainly found in postmenopausal women, generally have a good prognosis and arise from atypical endometrial hyperplasia, which is thought to be caused by long term unopposed estrogenic stimulation[50]. Type I carcinomas are frequently associated with mutations in the *PTEN* tumor suppressor gene, the *KRAS* oncogene and the WNT/β-catenin signaling pathway[51-53]. Next to type I, about 10% of all endometrial cancers are type II carcinomas. By definition, these tumors are either poorly differentiated endometrioid or non-endometrioid carcinomas, of which serous endometrial carcinoma is the most aggressive. Type II tumors are more common in premenopausal women and are not caused by unopposed estrogen exposure, but are associated with endometrial atrophy and, in case of serous carcinoma, associated with endometrial intra-epithelial carcinoma (EIC)[50, 54]. Furthermore, in type II endometrial cancers, myometrial and vascular invasion are more commonly found and patients are at high risk of recurrence and metastatic disease[33]. Mutations associated with type II endometrial carcinoma are found in *ERBB-2* (*HER2/NEU*) and *TP53*[55, 56]. Interestingly, as in serous ovarian cancer, serous endometrial carcinomas show nuclear accumulation of mutant P53[57].

## Treatment and prognosis

Following initial diagnosis, surgery is the cornerstone of treatment and hysterectomy (either alone or in combination with bilateral salpingo-oophorectomy and/or lymphadenectomy) by laparoscopy or laparotomy is an adequate treatment in most cases with a 7-year survival rate of 80%[33]. Where there is recurrent or high stage metastatic disease, however, the situation is very different and 5-year survival drops to 17%. Here, (neo)adjuvant radiation and/or systemic therapy in combination with surgery is indicated and in general, progressive disease has a poor prognosis accounting for 74.000 deaths worldwide each year (2,2 percent of all cancer related death in women)[31, 33]. Important prognostic factors for recurrent and metastatic disease include FIGO stage, tumor grade, age at diagnosis, depth of myometrial invasion, lymphovascular invasion, immunological T-cell distribution and estrogen and progesterone receptor status[58-68]. In addition, even though type II endometrial cancer only accounts for 10% of all endometrial cancer patients, more than 50% of all endometrial cancer recurrences and deaths are related to type II disease[69].

Because progesterone induced differentiation is thought to antagonize estrogen induced endometrial proliferation, progesterone (as medroxyprogesterone acetate, MPA) is used in palliative treatment of advanced and recurrent endometrial cancer with modest response-rates (15-25%)[70]. Furthermore, MPA is used as a primary treatment for atypical endometrial hyperplasia

and well differentiated endometrial carcinoma in premenopausal women determined to preserve fertility. Here, response-rates can be up to 60%, indicating that progesterone signaling is a potent inhibitor of carcinogenesis[71, 72].

### Tumor infiltrating T-lymphocytes and endometrial cancer

Infiltrating solid tumor growth is thought to cause an inflammatory response similar to an acute injury, which eventually results in infiltration of T-lymphocytes[73]. In several types of cancer, such as melanoma, colorectal cancer, ovarian cancer and cervical cancer, the presence of these tumor-infiltrating T-lymphocytes (TILs) has been extensively investigated and is associated with improved prognosis and reduced cancer recurrence[74-80]. In endometrial cancer, infiltration of cytotoxic (CD8+) T-lymphocytes within the tumor was positively correlated with improved disease free and overall survival[59, 64]. Furthermore, as in ovarian cancer, a high cytotoxic/regulatory (CD8+/FOXP3+) T-lymphocyte ratio was found to be associated with improved survival in type 1 endometrial cancer[59]. In addition, low numbers of FOXP3+ T-lymphocytes were correlated with low vascular density and estrogen receptor negativity, which are associated with improved endometrial cancer prognosis[81]. However, the underling mechanisms by which TILs influence endometrial cancer survival and recurrence is not understood.

### WNT/β-catenin signaling and endometrial cancer

As described earlier, the WNT/β-catenin signaling pathway plays a rate-limiting role in maintenance and control of the endometrium where it regulates the fine balance between proliferation (WNT-on) and differentiation (WNT-off) under influence of estrogen and progesterone. Therefore, a causal role for WNT/β-catenin signaling in endometrial carcinogenesis was proposed. This role was confirmed by the frequent finding of gene mutations in endometrial cancer, that can lead to constitutive activation of canonical WNT/β-catenin signaling[28, 82-86]. In agreement to this, as measured by nuclear β-catenin accumulation, approximately 40% of well differentiated endometrioid adenocarcinomas actually show high levels of WNT/β-catenin signaling[24, 87, 88]. As indicated earlier, progesterone induced inhibition of the WNT/β-catenin signaling pathway, for example by upregulation of *DKK1* and *FOXO1*, was found to reduce endometrial cancer progression[28, 89]. Next to these more clinical findings a number of mice models, which are extensively described in chapter 2 of this thesis, also indicate a causal relationship between activated WNT/β-catenin signaling and endometrial carcinogenesis[90-92].

## Ovarian cancer

Every year, worldwide, approximately 225.000 women are diagnosed with ovarian cancer, accounting for 3,7% of all cancers found in women. Although this incidence is relatively low, ovarian cancer accounts for 140.000 deaths each year, making it the most lethal gynecological malignancy[31]. In the Netherlands, each year, approximately 1200 patients are diagnosed with ovarian cancer, accounting for an incidence of 14,3 per 100.000 women and a cumulative risk of endometrial cancer up to 75 years of age of 0,95%[31].

## Risk factors

Because of the high mortality of ovarian cancer, the identification of risk factors is of vital importance. The most important risk factors are ovarian cancer specific genetic syndromes such as the hereditary breast-ovarian cancer syndrome (*BRCA1* and *BRCA2* gene mutations) and Lynch syndrome (*MLH1*, *MSH2* and *MSH6* gene mutations). The estimated lifetime risk for ovarian cancer is 35-46 percent for *BRCA1* mutation carriers and 13-23 percent for *BRCA2* mutation carriers. Because of this high risk and since *BRCA* mutations are mainly associated with high grade serous ovarian cancer, risk-reducing or prophylactic bilateral salpingo-oophorectomy is offered as preventive treatment[93, 94]. Other risk factors include endometriosis and factors involved with a high number of ovulations, such as: null parity, delayed childbearing, estrogen replacement therapy for more than five years, late menopause, early menarche and a high fat diet[95-99]. In contrast, factors that reduce the number of ovulations, such as oral contraceptive use, pregnancy and lactation, decrease the risk of ovarian cancer[99].

## Symptoms and diagnosis

The high mortality is mainly caused by the fact that approximately 64% of women with ovarian cancer are diagnosed at a late stage of disease (stage III or IV), where the disease has already spread throughout the abdomen[100]. This delayed diagnosis is mainly caused by two factors: firstly, the precursor lesion causing epithelial ovarian cancer is still debated amongst scientists and clinicians, making development of tools for early detection and targeted therapy difficult. Secondly, ovarian cancer shows late and unspecific symptoms such as fatigue, nausea, abdominal (pelvic) pain, bloating and feeling full, symptoms commonly present in many women and in many types of disease[101].

Diagnosis of ovarian cancer commonly includes measurement of the serum CA125 level and transvaginal ultrasonography, while internal gynecological examination is relatively sensitive for detecting ovarian masses[102]. CA125, encoded by *MUC16*, was discovered in the eighties and is the most frequently used biomarker for ovarian cancer. Elevated levels of serum CA125 are found in approximately 80% of patients with advanced ovarian cancer[103]. However, although a combination of CA125 level measurement and transvaginal ultrasonography is able to detect ovarian cancer at a relatively early stage, this does not improve clinical outcome and therefore routine ovarian cancer screening is not recommended[104, 105]. Furthermore, several other abdominal conditions, such as pelvic inflammatory disease, endometriosis, functional ovarian cysts, menstruation and pregnancy, can also result in increased CA125 levels[106]. Other biomarkers for ovarian carcinoma are serum measurement of HE4, either alone or in combination with CA125 (ROMA algorithm), and the biomarkerpanel OVA1 that includes serum measurement of CA125, β2-microglobulin, apolipoprotein, prealbumin and transferrin[107-109]. Even though ultrasound and biomarker tests are relatively good diagnostic tools, the final diagnosis of ovarian cancer is made during surgery.

## Pathology

Upon histological diagnosis, three major types of ovarian cancer can be distinguished: epithelial (85-95%), stromal (5-8%) and germ cell (3-5%)[110]. Epithelial ovarian cancer is most common in postmenopausal women and can be divided in four distinct subtypes: serous, endometrioid, mucinous and clear-cell ovarian cancer[110]. As in endometrial cancer, epithelial ovarian cancer can be further divided in two subgroups: type I and type II[111]. Type I tumors include 25% of all ovarian cancer cases, are slow growing, generally confined to the ovary, low grade and seem to develop from endometriosis or well-established borderline lesions. Mutations associated with type I tumors are found in *PTEN, KRAS*, *BRAF* and *CTNNB1*. Type II tumors account for 75% of all ovarian cancer cases, are characterized by fast growing, highly aggressive and rapidly spreading tumors and include high-grade serous carcinoma, carcinosarcomas and undifferentiated tumors. Genetic mutations associated with type II disease are generally found in *TP53*[111].

## The origin of ovarian cancer

For many decades the ovarian surface epithelium (OSE) was appointed as the only origin of epithelial ovarian cancer. Here, ovarian surface epithelial cells are thought to accumulated DNA mutations due to repeated ovulation-induced mechanical and chemotoxic damage, followed by entrapment of the OSE in a repaired ovulation site causing so called cortical inclusion cysts (CICs). Under the influence of the ovarian micro-environment and additional genetic disturbances, these CICs become metaplastic, obtain a Müllerian phenotype and eventually become malignant[101].

Over the last decade, however, many researchers questioned this hypothesis for the following reasons. Firstly the three most important epithelial ovarian subtypes strongly represent Müllerian duct derived structures, while the OSE does not display these characteristics: serous ovarian cancer resembles the epithelium of the fallopian tube; endometrioid ovarian cancer shows similarity to endometrial glands; and mucinous ovarian cancer resembles the endocervical epithelium[112]. Secondly, pathways and genes involved in Müllerian duct development such as WNT/β-catenin signaling, *HOX*-genes and *PAX*-genes, are highly expressed in ovarian cancer but not in the OSE[113-121]. Thirdly, upon review of fallopian tubes, early benign (P53 signatures), intermediate (serous tubal intra-epithelial lesions, STILs) and malignant (serous tubal intra-epithelial carcinomas, STICs) lesions were identified in patients at risk for or with a concurrent serous ovarian carcinoma[122-130]. Interestingly, these malignant STICs showed similar histological and genetical characteristics as concurrent serous ovarian cancer, which indicates a causal relationship[125, 126]. Fourthly, frequently used ovarian cancer biomarkers such as CA125, PAX2 and WT1 are expressed by Müllerian duct derived structures, but not in the OSE[116, 119, 131, 132]. Finally our group was able to show that a population of stem-like cells is located in the distal and fimbriae part of the fallopian tube (near the ovary) in mice, but not the OSE[133]. Upon isolation, these cells formed spheroids capable of self-renewal and fetal calf serum (FCS) stimulation initiated differentiation of these cells into gland-like structures with a clear Müllerian phenotype. Hence, due to their Müllerian characteristics and close proximity to the ovary, it was hypothesized that these stem-like cells may seize ovulation induced

DNA damage causing them to transform into malignant STICs, and initiate ovarian cancer[133].
Based on these and other findings more extensively discussed in chapter 4 of this thesis, a different origin of epithelial ovarian cancer was proposed: tissues derived from the Müllerian duct. Unfortunately, good animal models aiming to confirm this hypothesis are still lacking.

### Treatment and prognosis

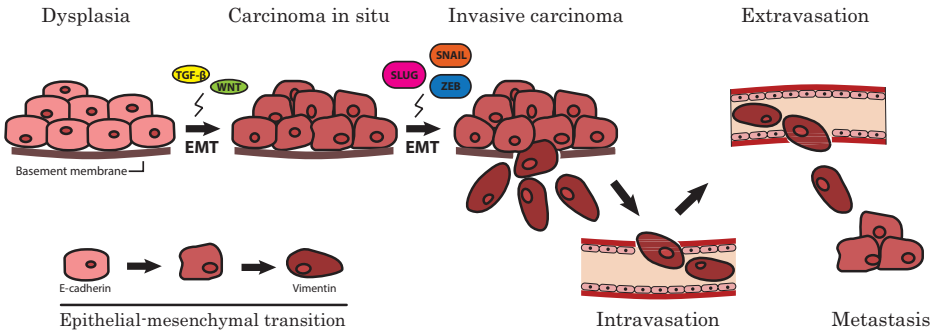
The treatment of ovarian cancer consists of two pillars: tumor debulking surgery and (neo)adjuvant chemotherapy. Surgical treatment involves total hysterectomy, bilateral salpingo-oophorectomy, pelvic and paraaortic lymfadenectomy and removal of the omentum. As described before, during surgery the final diagnosis is made and the tumor is staged. However, outcome of treatment is highly dependent on the type, stage at diagnosis and the histological grade, with high stage and poor cell differentiation (high grade) corresponding with poor prognosis[100]. Because in most patients microscopic disease is still present after surgery, chemotherapy is an important part of the treatment. Unfortunately, even though initially most tumors respond well, eventually chemoresistant disease will develop and as a result, in the Netherlands overall survival of ovarian cancer patients is only approximately 41% and in total almost 69% of patients die from the disease[100]. Even more devastating, five year survival of the most frequently diagnosed stage III and IV disease is only 28,6 and 14,1%, respectively[100].

## Cancer progression: epithelial to mesenchymal transition

Epithelial cells are virtually incapable of migration, due to their strong cell-cell bindings, mediated for example by E-cadherin, and the presence of the basement membranes. Migration of epithelial cells, however, is vital during the most crucial steps of embryogenesis and to circumvent this problem, epithelial cells are capable of transition into a more mesenchymal phenotype[134]. Unfortunately, this transition of an epithelial phenotype towards a more mesenchymal phenotype also acts as a subsequent step in progression from a confined tumor to invasive and metastatic disease.
Central to epithelial to mesenchymal transition (EMT) is the activation of important signaling pathways such as WNT/β-catenin, FGF, EGF and TGF-β[134]. Activation of these pathways results in induction of EMT transcription factors such as SNAIL1, SLUG, ZEB1/2, TWIST1/2, GOOSEGOID and KLF8. Upon expression, SNAIL1, SLUG, KLF8 and ZEB1/2 directly repress the activity of the *E-cadherin* promotor, while TWIST1/2 and GOOSEGOID repress E-cadherin indirectly[134-136]. In addition to the repression of epithelial E-cadherin, EMT transcription factors cause gain of mesenchymal markers such as vimentin and N-cadherin[134]. Next to downregulation of E-cadherin and upregulation of vimentin and N-cadherin, expression of SNAIL1 and ZEB1/2 also induces matrix metalloproteinases (MMP), causing degradation of the basement membrane, thereby facilitating invasion[137-139]. Furthermore, SNAIL1 and ZEB1 inhibit epithelial polarity by repression of *PAR*, *CRUMBS3* and *SCRIBBLE*[140, 141].

Eventually, EMT enables migration, invasion, intravasation, dissemination and extravasation of tumor cells resulting in widespread metastasis (Fig. 3)[142]. In addition to metastasis, EMT is also an important factor in resistance to cell death and senescence, chemo and immunotherapy and anti-tumor immune response, and in induction of stem-like cell properties[134].

**Figure 3: Epithelial to mesenchymal transtion (EMT) in cancer progression.**




Upon activation, transition from an epithelial towards a more mesenchymal phenotype (EMT) enables migration, invasion, intravasation and extravasation of tumor cells, which can result in widespread metastasis. Figure adapted from Thiery (2002) Nat Rev Cancer;2:442-54.

## Aims of the thesis

The main goal of the work presented in this thesis was to unravel the mechanisms involved in initiation and progression of Müllerian duct derived malignancies. For this purpose, three research questions were posed:

1. What is the effect of progesterone receptor signaling on the tumor specific immune response, epithelial-to-mesenchymal transition and recurrence in endometrial cancer?
2. What is the effect of activation of WNT/β-catenin signaling on Müllerian duct derived tissues?
3. Are Müllerian duct derived tissues the origin of epithelial ovarian cancer; can we initiate ovarian cancer from these tissues; and can we identify and characterize tubal precursor lesions of serous ovarian carcinoma in controls, patients susceptible for and patients with serous ovarian cancer?

## Outline of the thesis

The WNT/β-catenin signaling pathway plays a rate-limiting role in the development of many organs and is of great importance in tissue development and homeostasis during adult live. **Chapter 2** reviews the role of WNT/β-catenin signaling on the Müllerian-derived female reproductive tract, especially focusing on its interaction with sex hormones during uterine development, pregnancy, endometriosis and cancer. Since sex hormones were shown to interact with important pathways involved in cancer initiation and development, the role of progesterone receptor signaling on endometrial carcinoma was assessed in **Chapter 3**. In this study, using endometrial cancer cell lines and patient tissue specimens, the role of progesterone receptor signaling on endometrial cancer triggered immune response, cell migration, recurrence, and metastasis was investigated.
Early detection of ovarian cancer is hampered by the fact that the origin of ovarian cancer is still debated. Over the last decades, researchers have proposed the hypothesis that epithelial ovarian cancer originates from Müllerian derived structures and current perspectives on this Müllerian origin of epithelial ovarian cancer are introduced and discussed in **Chapter 4**. Knowing that in a high percentage of endometrioid ovarian cancers WNT/β-catenin signaling is activated, and in view of the hypothesis that ovarian cancer may originate from the distal oviduct, in **Chapter 5** we have documented an endometrioid ovarian cancer mouse model using conditional activation of WNT/β-catenin signaling in Müllerian duct derived tissues. The role of Müllerian duct derived tissues in epithelial ovarian cancer initiation and progression is further assessed for the human situation in **Chapter 6**. Here we have investigated the prevalence of tubal precursor lesions of serous ovarian cancer in different patient populations, studied the molecular and migratory characteristics of the observed lesions and compared them to concurrent serous ovarian tumor.
**Chapter 7 and 8** provide a summary of the results of the studies in this thesis and a general discussion. Furthermore, directions for future research and possible clinical implications are assessed.

# References


1. Acien P. Embryological observations on the female genital tract. *Hum Reprod* 1992;**7:**437-45.
2. Carlson BM. *Human Embryology and Developmental Biology.* 3 ed: Mosby; 2004.
3. Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. *Physiol Rev* 2007;**87:**1-28.
4. Ikeda Y, Takeda Y, Shikayama T, Mukai T, Hisano S, et al. Comparative localization of Dax-1 and Ad4BP/SF-1 during development of the hypothalamic-pituitary-gonadal axis suggests their closely related and distinct functions. *Dev Dyn* 2001;**220:**363-76.
5. Guioli S, Sekido R, Lovell-Badge R. The origin of the Mullerian duct in chick and mouse. *Dev Biol* 2007;**302:**389-98.
6. Kobayashi A, Shawlot W, Kania A, Behringer RR. Requirement of Lim1 for female reproductive tract development. *Development* 2004;**131:**539-49.
7. Masse J, Watrin T, Laurent A, Deschamps S, Guerrier D, et al. The developing female genital tract: from genetics to epigenetics. *Int J Dev Biol* 2009;**53:**411-24.
8. Carroll TJ, Park JS, Hayashi S, Majumdar A, McMahon AP. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev Cell* 2005;**9:**283-92.
9. Orvis GD, Behringer RR. Cellular mechanisms of Mullerian duct formation in the mouse. *Dev Biol* 2007;**306:**493-504.
10. Mericskay M, Kitajewski J, Sassoon D. Wnt5a is required for proper epithelial-mesenchymal interactions in the uterus. *Development* 2004;**131:**2061-72.
11. Miller C, Sassoon DA. Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. *Development* 1998;**125:**3201-11.
12. Podlasek C, Houston J, McKenna KE, McVary KT. Posterior Hox gene expression in developing genitalia. *Evol Dev* 2002;**4:**142-63.
13. Heineman MJ, Evers JLH, Massuger LFAG, Steegers EAP. *Obstetrie en Gynaecologie.* 6 ed. Maarssen: Elsevier Gezondheidszorg; 2007.
14. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med* 2007;**25:**445-53.
15. Jungueira LC, Carneiro J. *Basic Histology.* 11 ed: McGraw-Hill; 2005.
16. Ryan KJ. Biochemistry of aromatase: significance to female reproductive physiology. *Cancer Res* 1982;**42:**3342s-4s.
17. Boron WF, Boulpaep EL. *Medical Physiology.* Updated ed. Philadelphia: Elsevier Saunders; 2005.
18. Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 1982;**31:**99-109.
19. Clevers H. Wnt/[beta]-Catenin Signaling in Development and Disease. *Cell* 2006;**127:**469-80.
20. Nusse R. The Wnt Homepage. 2013 [cited; Available from: www.stanford.edu/group/nusselab/cgi-bin/wnt/
21. Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 2000;**407:**535-8.
22. Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, et al. Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* 1998;**280:**596-9.
23. Daniels DL, Weis WI. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat Struct Mol Biol* 2005;**12:**364-71.
24. Nei H, Saito T, Yamasaki H, Mizumoto H, Ito E, et al. Nuclear localization of beta-catenin in normal and carcinogenic endometrium. *Mol Carcinog* 1999;**25:**207-18.
25. Hou X, Tan Y, Li M, Dey SK, Das SK. Canonical Wnt signaling is critical to estrogen-mediated uterine growth. *Mol Endocrinol* 2004;**18:**3035-49.
26. Cloke B, Huhtinen K, Fusi L, Kajihara T, Yliheikkila M, et al. The androgen and progesterone receptors regulate distinct gene networks and cellular functions in decidualizing endometrium. *Endocrinology* 2008;**149:**4462-74.

27. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* 2006;**147:**1097-121.
28. Wang Y, Hanifi-Moghaddam P, Hanekamp EE, Kloosterboer HJ, Franken P, et al. Progesterone inhibition of Wnt/beta-catenin signaling in normal endometrium and endometrial cancer. *Clin Cancer Res* 2009;**15:**5784-93.
29. Hanifi-Moghaddam P, Boers-Sijmons B, Klaassens AH, van Wijk FH, den Bakker MA, et al. Molecular analysis of human endometrium: short-term tibolone signaling differs significantly from estrogen and estrogen + progestagen signaling. *J Mol Med (Berl)* 2007;**85:**471-80.
30. Klaassens AH, van Wijk FH, Hanifi-Moghaddam P, Sijmons B, Ewing PC, et al. Histological and immunohistochemical evaluation of postmenopausal endometrium after 3 weeks of treatment with tibolone, estrogen only, or estrogen plus progestagen. *Fertil Steril* 2006;**86:**352-61.
31. Ferlay J SH, Bray F, Forman D, Mathers C and Parkin DM. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. [cited; Available from: http://globocan.iarc.fr, accessed on day/month/year.
32. Howlader N, Noone AM, Krapcho M, Garshell J, N. N, et al. SEER Cancer Statistics Review 1975-2010. November 2012 [cited; Available from: http://seer.cancer.gov/csr/1975_2010/
33. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, et al. Endometrial cancer. *Lancet* 2005;**366:**491-505.
34. Bianchini F, Kaaks R, Vainio H. Overweight, obesity, and cancer risk. *The Lancet Oncology* 2002;**3:**565-74.
35. Weiderpass E, Persson I, Adami HO, Magnusson C, Lindgren A, et al. Body size in different periods of life, diabetes mellitus, hypertension, and risk of postmenopausal endometrial cancer (Sweden). *Cancer Causes Control* 2000;**11:**185-92.
36. Klip H, Burger CW, Kenemans P, van Leeuwen FE. Cancer risk associated with subfertility and ovulation induction: a review. *Cancer Causes Control* 2000;**11:**319-44.
37. Schouten LJ, Goldbohm RA, van den Brandt PA. Anthropometry, physical activity, and endometrial cancer risk: results from the Netherlands cohort study. *Int J Gynecol Cancer* 2006;**16 Suppl 2:**492.
38. Van Gorp T, Neven P. Endometrial safety of hormone replacement therapy: review of literature. *Maturitas* 2002;**42:**93-104.
39. Bergman L, Beelen ML, Gallee MP, Hollema H, Benraadt J, et al. Risk and prognosis of endometrial cancer after tamoxifen for breast cancer. Comprehensive Cancer Centres' ALERT Group. Assessment of Liver and Endometrial cancer Risk following Tamoxifen. *Lancet* 2000;**356:**881-7.
40. Gruber SB, Thompson WD. A population-based study of endometrial cancer and familial risk in younger women. Cancer and Steroid Hormone Study Group. *Cancer Epidemiol Biomarkers Prev* 1996;**5:**411-7.
41. Hemminki K, Granstrom C. Familial clustering of ovarian and endometrial cancers. *Eur J Cancer* 2004;**40:**90-5.
42. Lynch HT, Shaw MW, Magnuson CW, Larsen AL, Krush AJ. Hereditary factors in cancer. Study of two large midwestern kindreds. *Arch Intern Med* 1966;**117:**206-12.
43. Pennington KP, Walsh T, Lee M, Pennil C, Novetsky AP, et al. BRCA1, TP53, and CHEK2 germline mutations in uterine serous carcinoma. *Cancer* 2013;**119:**332-8.
44. Hinkula M, Pukkala E, Kyyronen P, Kauppila A. Grand multiparity and incidence of endometrial cancer: a population-based study in Finland. *Int J Cancer* 2002;**98:**912-5.
45. Horn-Ross PL, John EM, Canchola AJ, Stewart SL, Lee MM. Phytoestrogen intake and endometrial cancer risk. *J Natl Cancer Inst* 2003;**95:**1158-64.
46. Lesko SM, Rosenberg L, Kaufman DW, Helmrich SP, Miller DR, et al. Cigarette smoking and the risk of endometrial cancer. *N Engl J Med* 1985;**313:**593-6.
47. Deligeoroglou E, Michailidis E, Creatsas G. Oral contraceptives and reproductive system cancer. *Ann N Y Acad Sci* 2003;**997:**199-208.
48. Clark TJ. Outpatient hysteroscopy and ultrasonography in the management of endometrial disease. *Curr Opin Obstet Gynecol* 2004;**16:**305-11.
49. Clement PB, Young RH. Endometrioid carcinoma of the uterine corpus: a review of its pathology with emphasis on recent advances and problematic aspects. *Adv Anat Pathol* 2002;**9:**145-84.
50. Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol* 1983;**15:**10-7.

51. Enomoto T, Inoue M, Perantoni AO, Buzard GS, Miki H, et al. K-ras activation in premalignant and malignant epithelial lesions of the human uterus. *Cancer Res* 1991;**51:**5308-14.
52. Kong D, Suzuki A, Zou TT, Sakurada A, Kemp LW, et al. PTEN1 is frequently mutated in primary endometrial carcinomas. *Nat Genet* 1997;**17:**143-4.
53. van der Horst PH, Wang Y, van der Zee M, Burger CW, Blok LJ. Interaction between sex hormones and WNT/beta-catenin signal transduction in endometrial physiology and disease. *Mol Cell Endocrinol* 2012;**358:**176-84.
54. Ambros RA, Sherman ME, Zahn CM, Bitterman P, Kurman RJ. Endometrial intraepithelial carcinoma: a distinctive lesion specifically associated with tumors displaying serous differentiation. *Hum Pathol* 1995;**26:**1260-7.
55. Okamoto A, Sameshima Y, Yamada Y, Teshima S, Terashima Y, et al. Allelic loss on chromosome 17p and p53 mutations in human endometrial carcinoma of the uterus. *Cancer Res* 1991;**51:**5632-5.
56. Santin AD. HER2/neu overexpression: has the Achilles' heel of uterine serous papillary carcinoma been exposed? *Gynecol Oncol* 2003;**88:**263-5.
57. Wheeler DT, Bell KA, Kurman RJ, Sherman ME. Minimal uterine serous carcinoma: diagnosis and clinicopathologic correlation. *Am J Surg Pathol* 2000;**24:**797-806.
58. Cohn DE, Horowitz NS, Mutch DG, Kim SM, Manolitsas T, et al. Should the presence of lymphvascular space involvement be used to assign patients to adjuvant therapy following hysterectomy for unstaged endometrial cancer? *Gynecol Oncol* 2002;**87:**243-6.
59. de Jong RA, Leffers N, Boezen HM, ten Hoor KA, van der Zee AG, et al. Presence of tumor-infiltrating lymphocytes is an independent prognostic factor in type I and II endometrial cancer. *Gynecol Oncol* 2009;**114:**105-10.
60. Ehrlich CE, Young PC, Stehman FB, Sutton GP, Alford WM. Steroid receptors and clinical outcome in patients with adenocarcinoma of the endometrium. *Am J Obstet Gynecol* 1988;**158:**796-807.
61. Hanekamp EE, Gielen SC, Smid-Koopman E, Kuhne LC, de Ruiter PE, et al. Consequences of loss of progesterone receptor expression in development of invasive endometrial cancer. *Clin Cancer Res* 2003;**9:**4190-9.
62. Jeon YT, Park IA, Kim YB, Kim JW, Park NH, et al. Steroid receptor expressions in endometrial cancer: clinical significance and epidemiological implication. *Cancer Lett* 2006;**239:**198-204.
63. Jolly S, Vargas CE, Kumar T, Weiner SA, Brabbins DS, et al. The impact of age on long-term outcome in patients with endometrial cancer treated with postoperative radiation. *Gynecol Oncol* 2006;**103:**87-93.
64. Kondratiev S, Sabo E, Yakirevich E, Lavie O, Resnick MB. Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. *Clin Cancer Res* 2004;**10:**4450-6.
65. Kosary CL. FIGO stage, histology, histologic grade, age and race as prognostic factors in determining survival for cancers of the female gynecological system: an analysis of 1973-87 SEER cases of cancers of the endometrium, cervix, ovary, vulva, and vagina. *Semin Surg Oncol* 1994;**10:**31-46.
66. Lindauer J, Fowler JM, Manolitsas TP, Copeland LJ, Eaton LA, et al. Is there a prognostic difference between depth of myometrial invasion and the tumor-free distance from the uterine serosa in endometrial cancer? *Gynecol Oncol* 2003;**91:**547-51.
67. van der Horst PH, Wang YY, Vandenput I, Kuhne LC, Ewing PC, et al. Progesterone Inhibits Epithelial-to-Mesenchymal Transition in Endometrial Cancer. *PLoS One* 2012;**7**.
68. Pecorelli S. Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. *Int J Gynaecol Obstet* 2009;**105:**103-4.
69. Creasman WT, Kohler MF, Odicino F, Maisonneuve P, Boyle P. Prognosis of papillary serous, clear cell, and grade 3 stage I carcinoma of the endometrium. *Gynecol Oncol* 2004;**95:**593-6.
70. Thigpen JT, Brady MF, Alvarez RD, Adelson MD, Homesley HD, et al. Oral medroxyprogesterone acetate in the treatment of advanced or recurrent endometrial carcinoma: a dose-response study by the Gynecologic Oncology Group. *J Clin Oncol* 1999;**17:**1736-44.
71. Kim YB, Holschneider CH, Ghosh K, Nieberg RK, Montz FJ. Progestin alone as primary treatment of endometrial carcinoma in premenopausal women. Report of seven cases and review of the literature. *Cancer* 1997;**79:**320-7.
72. Yahata T, Fujita K, Aoki Y, Tanaka K. Long-term conservative therapy for endometrial adenocarcinoma in young women. *Hum Reprod* 2006;**21:**1070-5.

73. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, et al. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res* 2007;**67:**9518-27.
74. Clemente CG, Mihm MC, Jr., Bufalino R, Zurrida S, Collini P, et al. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 1996;**77:**1303-10.
75. de Vos van Steenwijk PJ, Heusinkveld M, Ramwadhdoebe TH, Lowik MJ, van der Hulst JM, et al. An unexpectedly large polyclonal repertoire of HPV-specific T cells is poised for action in patients with cervical cancer. *Cancer Res* 2010;**70:**2707-17.
76. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;**313:**1960-4.
77. Kilic A, Landreneau RJ, Luketich JD, Pennathur A, Schuchert MJ. Density of tumor-infiltrating lymphocytes correlates with disease recurrence and survival in patients with large non-small-cell lung cancer tumors. *J Surg Res* 2011;**167:**207-10.
78. Le DT, Ladle BH, Lee T, Weiss V, Yao X, et al. CD8(+) Foxp3(+) tumor infiltrating lymphocytes accumulate in the context of an effective anti-tumor response. *Int J Cancer* 2011;**129:**636-47.
79. Leffers N, Fehrmann RS, Gooden MJ, Schulze UR, Ten Hoor KA, et al. Identification of genes and pathways associated with cytotoxic T lymphocyte infiltration of serous ovarian cancer. *Br J Cancer* 2010;**103:**685-92.
80. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 2003;**348:**203-13.
81. Giatromanolaki A, Bates GJ, Koukourakis MI, Sivridis E, Gatter KC, et al. The presence of tumor-infiltrating FOXP3+ lymphocytes correlates with intratumoral angiogenesis in endometrial cancer. *Gynecol Oncol* 2008;**110:**216-21.
82. Hecht JL, Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis. *J Clin Oncol* 2006;**24:**4783-91.
83. Konopka B, Janiec-Jankowska A, Czapczak D, Paszko Z, Bidzinski M, et al. Molecular genetic defects in endometrial carcinomas: microsatellite instability, PTEN and beta-catenin (CTNNB1) genes mutations. *J Cancer Res Clin Oncol* 2007;**133:**361-71.
84. Moreno-Bueno G, Hardisson D, Sanchez C, Sarrio D, Cassia R, et al. Abnormalities of the APC/beta-catenin pathway in endometrial cancer. *Oncogene* 2002;**21:**7981-90.
85. Pijnenborg JM, Kisters N, van Engeland M, Dunselman GA, de Haan J, et al. APC, beta-catenin, and E-cadherin and the development of recurrent endometrial carcinoma. *Int J Gynecol Cancer* 2004;**14:**947-56.
86. Fukuchi T, Sakamoto M, Tsuda H, Maruyama K, Nozawa S, et al. Beta-catenin mutation in carcinoma of the uterine endometrium. *Cancer Res* 1998;**58:**3526-8.
87. Saegusa M, Okayasu I. Frequent nuclear beta-catenin accumulation and associated mutations in endometrioid-type endometrial and ovarian carcinomas with squamous differentiation. *J Pathol* 2001;**194:**59-67.
88. Scholten AN, Creutzberg CL, van den Broek LJ, Noordijk EM, Smit VT. Nuclear beta-catenin is a molecular feature of type I endometrial carcinoma. *J Pathol* 2003;**201:**460-5.
89. Ward EC, Hoekstra AV, Blok LJ, Hanifi-Moghaddam P, Lurain JR, et al. The regulation and function of the forkhead transcription factor, Forkhead box O1, is dependent on the progesterone receptor in endometrial carcinoma. *Endocrinology* 2008;**149:**1942-50.
90. Tanwar PS, Lee HJ, Zhang L, Zukerberg LR, Taketo MM, et al. Constitutive activation of Beta-catenin in uterine stroma and smooth muscle leads to the development of mesenchymal tumors in mice. *Biol Reprod* 2009;**81:**545-52.
91. Tanwar PS, Zhang L, Roberts DJ, Teixeira JM. Stromal deletion of the APC tumor suppressor in mice triggers development of endometrial cancer. *Cancer Res* 2011;**71:**1584-96.
92. van der Zee M, Jia Y, Wang Y, Heijmans-Antonissen C, Ewing PC, et al. Alterations in Wnt/beta-catenin and Pten signaling play distinct roles in endometrial cancer initiation and progression. *J Pathol* 2013.
93. Chen S, Parmigiani G. Meta-analysis of BRCA1 and BRCA2 penetrance. *J Clin Oncol* 2007;**25:**1329-33.
94. Lakhani SR, Manek S, Penault-Llorca F, Flanagan A, Arnout L, et al. Pathology of ovarian cancers in BRCA1 and BRCA2 carriers. *Clin Cancer Res* 2004;**10:**2473-81.
95. Folsom AR, Anderson JP, Ross JA. Estrogen replacement therapy and ovarian cancer. *Epidemiology* 2004;**15:**100-4.

96. Prentice RL, Thomson CA, Caan B, Hubbell FA, Anderson GL, et al. Low-fat dietary pattern and cancer incidence in the Women's Health Initiative Dietary Modification Randomized Controlled Trial. *J Natl Cancer Inst* 2007;**99:**1534-43.

97. Vo C, Carney ME. Ovarian cancer hormonal and environmental risk effect. *Obstet Gynecol Clin North Am* 2007;**34:**687-700, viii.

98. Whiteman DC, Siskind V, Purdie DM, Green AC. Timing of pregnancy and the risk of epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 2003;**12:**42-6.

99. Whittemore AS. Characteristics relating to ovarian cancer risk: implications for prevention and detection. *Gynecol Oncol* 1994;**55:**S15-9.

100. van Altena AM, Karim-Kos HE, de Vries E, Kruitwagen RF, Massuger LF, et al. Trends in therapy and survival of advanced stage epithelial ovarian cancer patients in the Netherlands. *Gynecol Oncol* 2012;**125:**649-54.

101. Cannistra SA. Cancer of the Ovary. *New England Journal of Medicine* 2004;**351:**2519-29.

102. Goff BA, Mandel LS, Drescher CW, Urban N, Gough S, et al. Development of an ovarian cancer symptom index: possibilities for earlier detection. *Cancer* 2007;**109:**221-7.

103. Bast RC, Jr., Klug TL, St John E, Jenison E, Niloff JM, et al. A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. *N Engl J Med* 1983;**309:**883-7.

104. Force USPST. Screening for ovarian cancer: recommendation statement. U.S. Preventive Services Task Force. *Am Fam Physician* 2005;**71:**759-62.

105. Stirling D, Evans DG, Pichert G, Shenton A, Kirk EN, et al. Screening for familial ovarian cancer: failure of current protocols to detect ovarian cancer at an early stage according to the international Federation of gynecology and obstetrics system. *J Clin Oncol* 2005;**23:**5588-96.

106. Barney SP, Muller CY, Bradshaw KD. Pelvic masses. *Med Clin North Am* 2008;**92:**1143-61, xi.

107. Anastasi E, Marchei GG, Viggiani V, Gennarini G, Frati L, et al. HE4: a new potential early biomarker for the recurrence of ovarian cancer. *Tumour Biol* 2010;**31:**113-9.

108. Ueland FR, Desimone CP, Seamon LG, Miller RA, Goodrich S, et al. Effectiveness of a multivariate index assay in the preoperative assessment of ovarian tumors. *Obstet Gynecol* 2011;**117:**1289-97.

109. Van Gorp T, Cadron I, Despierre E, Daemen A, Leunen K, et al. HE4 and CA125 as a diagnostic test in ovarian cancer: prospective validation of the Risk of Ovarian Malignancy Algorithm. *Br J Cancer* 2011;**104:**863-70.

110. Roett MA, Evans P. Ovarian cancer: an overview. *Am Fam Physician* 2009;**80:**609-16.

111. Kurman RJ, Shih Ie M. Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications. *Int J Gynecol Pathol* 2008;**27:**151-60.

112. Karst AM, Drapkin R. Ovarian cancer pathogenesis: a model in evolution. *J Oncol* 2010;**2010:**932371.

113. Bitler BG, Nicodemus JP, Li H, Cai Q, Wu H, et al. Wnt5a suppresses epithelial ovarian cancer by promoting cellular senescence. *Cancer Res* 2011;**71:**6184-94.

114. Gatcliffe TA, Monk BJ, Planutis K, Holcombe RF. Wnt signaling in ovarian tumorigenesis. *Int J Gynecol Cancer* 2008;**18:**954-62.

115. Merritt MA, Parsons PG, Newton TR, Martyn AC, Webb PM, et al. Expression profiling identifies genes involved in neoplastic transformation of serous ovarian cancer. *BMC Cancer* 2009;**9:**378.

116. Ozcan A, Liles N, Coffey D, Shen SS, Truong LD. PAX2 and PAX8 expression in primary and metastatic mullerian epithelial tumors: a comprehensive comparison. *Am J Surg Pathol* 2011;**35:**1837-47.

117. Peng C, Zhang X, Yu H, Wu D, Zheng J. Wnt5a as a predictor in poor clinical outcome of patients and a mediator in chemoresistance of ovarian cancer. *Int J Gynecol Cancer* 2011;**21:**280-8.

118. Steg A, Wang W, Blanquicett C, Grunda JM, Eltoum IA, et al. Multiple gene expression analyses in paraffin-embedded tissues by TaqMan low-density array: Application to hedgehog and Wnt pathway analysis in ovarian endometrioid adenocarcinoma. *J Mol Diagn* 2006;**8:**76-83.

119. Tong GX, Chiriboga L, Hamele-Bena D, Borczuk AC. Expression of PAX2 in papillary serous carcinoma of the ovary: immunohistochemical evidence of fallopian tube or secondary Mullerian system origin? *Mod Pathol* 2007;**20:**856-63.

120. Varma RR, Hector SM, Clark K, Greco WR, Hawthorn L, et al. Gene expression profiling of a clonal isolate of oxaliplatin-resistant ovarian carcinoma cell line A2780/C10. *Oncol Rep* 2005;**14:**925-32.

121. Yoshioka S, King ML, Ran S, Okuda H, MacLean JA, 2nd, et al. WNT7A regulates tumor growth and progression in ovarian cancer through the WNT/beta-catenin pathway. *Mol Cancer Res* 2012;**10:**469-82.

122. Callahan MJ, Crum CP, Medeiros F, Kindelberger DW, Elvin JA, et al. Primary fallopian tube malignancies in BRCA-positive women undergoing surgery for ovarian cancer risk reduction. *J Clin Oncol* 2007;**25:**3985-90.

123. Carcangiu ML, Peissel B, Pasini B, Spatti G, Radice P, et al. Incidental carcinomas in prophylactic specimens in BRCA1 and BRCA2 germ-line mutation carriers, with emphasis on fallopian tube lesions: report of 6 cases and review of the literature. *Am J Surg Pathol* 2006;**30:**1222-30.

124. Hirst JE, Gard GB, McIllroy K, Nevell D, Field M. High rates of occult fallopian tube cancer diagnosed at prophylactic bilateral salpingo-oophorectomy. *Int J Gynecol Cancer* 2009;**19:**826-9.

125. Kindelberger DW, Lee Y, Miron A, Hirsch MS, Feltmate C, et al. Intraepithelial carcinoma of the fimbria and pelvic serous carcinoma: Evidence for a causal relationship. *Am J Surg Pathol* 2007;**31:**161-9.

126. Lee Y, Miron A, Drapkin R, Nucci MR, Medeiros F, et al. A candidate precursor to serous carcinoma that originates in the distal fallopian tube. *J Pathol* 2007;**211:**26-35.

127. Manchanda R, Abdelraheim A, Johnson M, Rosenthal AN, Benjamin E, et al. Outcome of risk-reducing salpingo-oophorectomy in BRCA carriers and women of unknown mutation status. *BJOG* 2011;**118:**814-24.

128. Medeiros F, Muto MG, Lee Y, Elvin JA, Callahan MJ, et al. The tubal fimbria is a preferred site for early adenocarcinoma in women with familial ovarian cancer syndrome. *Am J Surg Pathol* 2006;**30:**230-6.

129. Mingels MJ, Roelofsen T, van der Laak JA, de Hullu JA, van Ham MA, et al. Tubal epithelial lesions in salpingo-oophorectomy specimens of BRCA-mutation carriers and controls. *Gynecol Oncol* 2012;**127:**88-93.

130. Piek JM, van Diest PJ, Zweemer RP, Jansen JW, Poort-Keesom RJ, et al. Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer. *J Pathol* 2001;**195:**451-6.

131. Coppes MJ, Ye Y, Rackley R, Zhao XL, Liefers GJ, et al. Analysis of WT1 in granulosa cell and other sex cord-stromal tumors. *Cancer Res* 1993;**53:**2712-4.

132. Neunteufel W, Breitenecker G. Tissue expression of CA 125 in benign and malignant lesions of ovary and fallopian tube: a comparison with CA 19-9 and CEA. *Gynecol Oncol* 1989;**32:**297-302.

133. Wang Y, Sacchetti A, van Dijk MR, van der Zee M, van der Horst PH, et al. Identification of quiescent, stem-like cells in the distal female reproductive tract. *PLoS One* 2012;**7:**e40691.

134. Thiery JP, Acloque H, Huang RYJ, Nieto MA. Epithelial-Mesenchymal Transitions in Development and Disease. *Cell* 2009;**139:**871-90.

135. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* 2007;**7:**415-28.

136. Olmeda D, Jorda M, Peinado H, Fabra A, Cano A. Snail silencing effectively suppresses tumour growth and invasiveness. *Oncogene* 2007;**26:**1862-74.

137. Jorda M, Olmeda D, Vinyals A, Valero E, Cubillo E, et al. Upregulation of MMP-9 in MDCK epithelial cell line in response to expression of the Snail transcription factor. *J Cell Sci* 2005;**118:**3371-85.

138. Miyoshi A, Kitajima Y, Sumi K, Sato K, Hagiwara A, et al. Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. *Br J Cancer* 2004;**90:**1265-73.

139. Yokoyama K, Kamata N, Fujimoto R, Tsutsumi S, Tomonari M, et al. Increased invasion and matrix metalloproteinase-2 expression by Snail-induced mesenchymal transition in squamous cell carcinomas. *Int J Oncol* 2003;**22:**891-8.

140. Spaderna S, Schmalhofer O, Wahlbuhl M, Dimmler A, Bauer K, et al. The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. *Cancer Res* 2008;**68:**537-44.

141. Whiteman EL, Liu CJ, Fearon ER, Margolis B. The transcription factor snail represses Crumbs3 expression and disrupts apico-basal polarity complexes. *Oncogene* 2008;**27:**3875-9.

142. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002;**2:**442-54.

# Chapter 2

## Interaction between sexhormones and WNT/β-catenin signal transduction in endometrial physiology and disease

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## Abstract:

Wnt/β-catenin signalling plays a rate-limiting role in early development of many different organs in a broad spectrum of organisms. In the developing Müllerian duct, Wnt/β-catenin signalling is important for initiation, outgrowth, patterning and differentiation into vagina, cervix, uterus and oviducts. In adult life, sex hormones modulate Wnt/β-catenin signalling in the endometrium to maintain the monthly balance between estrogen-induced proliferation and progesterone-induced differentiation, and enhanced Wnt/β-catenin signalling seems to be involved in endometrial carcinogenesis. However, early in pregnancy enhanced Wnt/β-catenin signalling is prerequisite for proper implantation and invasion of trophoblast cells into endometrium and myometrium thus helping to form a placenta. Overall, it seems that tight control of Wnt/β-catenin signalling in time and space is important for initiation, development and normal function of the female reproductive tract. However, if Wnt/β-catenin signalling is not kept in check, it easily seems to initiate or contribute to development of a number of uterine disorders.

## General introduction:

Since the discovery of the proto-oncogene *Wnt1* in 1982, the Wnt signalling pathway has been shown to be a key regulator in development and disease[1, 2]. Currently, 20 secreted Wnt proteins have been identified that can bind to cell surface receptors of the Frizzled family[2]. Upon binding, three different pathways can be activated: the canonical Wnt/β-catenin signalling pathway[2], the non-canonical Wnt/Planar cell polarity pathway[3] or the Wnt/$Ca^{2+}$ pathway[4]. In this review, we will focus on canonical Wnt/β-catenin signalling in the female reproductive tract.

Central in activated canonical Wnt/β-catenin signalling is nuclear accumulation of β-catenin. Upon binding its ligand Wnt, the Frizzled receptor cooperates with a member of the LRP family[5]. As a result of this, via an interaction with a protein called dishevelled, the degradation complex (consisting of the scaffold proteins AXIN1 and AXIN2 (conductin), β-catenin (CTNNB1), the tumour suppressor APC (adenomatosis polyposis coli) and the Ser-Thr kinases CK1 (casein kinase I) and GSK3β (glycogen synthase kinase 3 beta)) dissociates and β-catenin is no longer targeted for degradation[6]. Stabilized β-catenin can now translocate to the nucleus where it displaces the transcription repressor Groucho (TLE), allowing members of the TCF/LEF transcription factor family to regulate Wnt target gene transcription[7]. For a thorough review on Wnt/β-catenin signalling, please visit: "The Wnt Homepage" (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/)[8].

## Wnt/β-catenin signalling in development of the Müllerian duct:

In early embryonic development in the anterior region of the coelomic cavity, Lim1 expressing epithelial cells are induced to invaginate by Wnt4, which is expressed from the mesonephros or coelomic epithelium[9]. Subsequently the primitive Müllerian duct anlage extends to and interacts with the Wolffian duct. This is followed by posterior elongation mediated by Wnt9b expressing epithelial cells from the Wolffian duct. In absence of the Wolffian duct or in case of absence of Wnt9b, the Müllerian duct does not develop further[10]. Outgrowth of the Müllerian duct is accomplished by proliferation of a group of coelomic epithelial cells resembling mesoepithelial cells at the distal tip[11, 12]. At the end of elongation both Müllerian ducts will fuse to form the uterovaginal tube, which joins the urogenital sinus. Once initiated, correct patterning of the Müllerian duct into vagina, cervix, uterus and oviducts partly depends on Wnt7a expressing epithelial cells of the oviduct and uterus and Wnt5a expressing mesenchymal cells of the uterus, cervix and vagina[13, 14].

In mice the Müllerian duct is formed around embryonic day 11.5, by an initial in-folding of Wnt4 expressing epithelial cells from the coelomic wall followed by posterior outgrowth to the cloacal region[9, 10]. Once the Müllerian duct is formed, Wnt4 is expressed at high levels by mesenchymal cells surrounding the duct. In *Wnt4* knockout animals a reversal of sexual development takes effect, exemplified by a testis-like appearance of the ovaries, absence of Müllerian structures and presence of Wolffian ducts. The absence of Müllerian ducts in both male and female *Wnt4* mutant mice during development indicates that Wnt4 is a prerequisite for the initial stages of Müllerian duct formation[15, 16]. Furthermore proper Wnt4 expression also seems necessary to suppress male differentiation in the female gonad.

Wnt9b is expressed in the Wolffian ducts during early embryonic stages when both Wolffian and Müllerian ducts are present (E9.5 – 14.5)[10]. In *Wnt9b*$^{-/-}$ embryos the Wolffian duct and the initial Müllerian anlage are present, but there is no extension of the Müllerian duct. This indicates that Wnt9b is necessary for posterior outgrowth during Müllerian duct formation[10].

Throughout the Müllerian duct epithelium Wnt7a is expressed before birth and in oviduct and uterine luminal epithelium after birth[14]. Targeted disruption of *Wnt7a* showed that oviducts were absent in most mice and, when present, remained uncoiled resembling uterus morphology. Furthermore, the uterus showed marked resemblance to the vagina with thickening of the surrounding musculature, a relatively thin stroma, pronounced loss of glands and a luminal epithelium with a clear squamous aspect. These data indicate that loss of Wnt7a seems to result in posteriorization of the female reproductive tract, indicating an important role for Wnt7a in correct patterning of the developing Müllerian duct[14, 17].

In normal mice, Wnt5a is expressed in mesenchymal cells surrounding the Müllerian duct and later in mesenchymal cells of uterus, cervix and vagina[18]. *Wnt5a* knockout female mice display normal oviducts and anterior uterine horns, but lack the more posterior cervical and vaginal structures. The uterine horns are severely coiled and either fused at midline or remain separated as blind ending pouches. Because *Wnt5a* mutant mice die at birth due to severe kidney problems, uterine tissues were grafted under the kidney capsule of immunodeficient mice. It was observed that in mutant grafts, gland formation was markedly impaired. Further investigations revealed that in wild type animals Wnt5a was highly expressed in the stromal region of the endometrium, and that Wnt5a and Wnt7a seem to act side by side to control gland formation[13].

In summary, the Wnt/β-catenin signalling pathway is important for initiation, outgrowth, patterning and differentiation of the Müllerian duct into vagina, cervix, uterus and oviduct (Table 1).

## Wnt/β-catenin signalling in uterine physiology:

The human uterus can be divided in 2 functional layers: the outer myometrial layer (myometrium) and the inner endometrial layer (endometrium). The endometrium is a dynamic tissue, which facilitates implantation, development and outgrowth of the embryo. The endometrium can also be divided in two layers: a functional and a basal layer. The functional layer, which is divested every month during menses, is replenished by the basal layer during the proliferative phase of the menstrual cycle. After menses during the first two weeks of the menstrual cycle estrogens, being produced by ovarian thecal cells, induce proliferation of the endometrium thus generating a new functional layer. During the second half of the menstrual cycle, the secretory phase, this functional layer will differentiate to prepare for implantation of the fertilized ovum. During this phase progesterone, which is produced by the corpus luteum, counterbalances estrogens proliferative effects and is responsible for the induction of differentiation[19] (Fig. 1).

In analogy to the situation in the gastrointestinal tract, where proliferating epithelial cells display activated Wnt/β-catenin signalling and differentiated cells show diminished Wnt/β-catenin signalling[2], a central role for Wnt/β-catenin signalling was hypothesized for the endometrium.

In short, during the proliferative phase of the menstrual cycle estrogens induce Wnt/β-catenin signalling. During the secretory phase of the menstrual cycle, however, progestagens counterbalance estrogen-induced proliferation by inhibition of Wnt/β-catenin signalling, thus inducing differentiation. Over time there have been multiple reports in literature which corroborate this hypothesis.

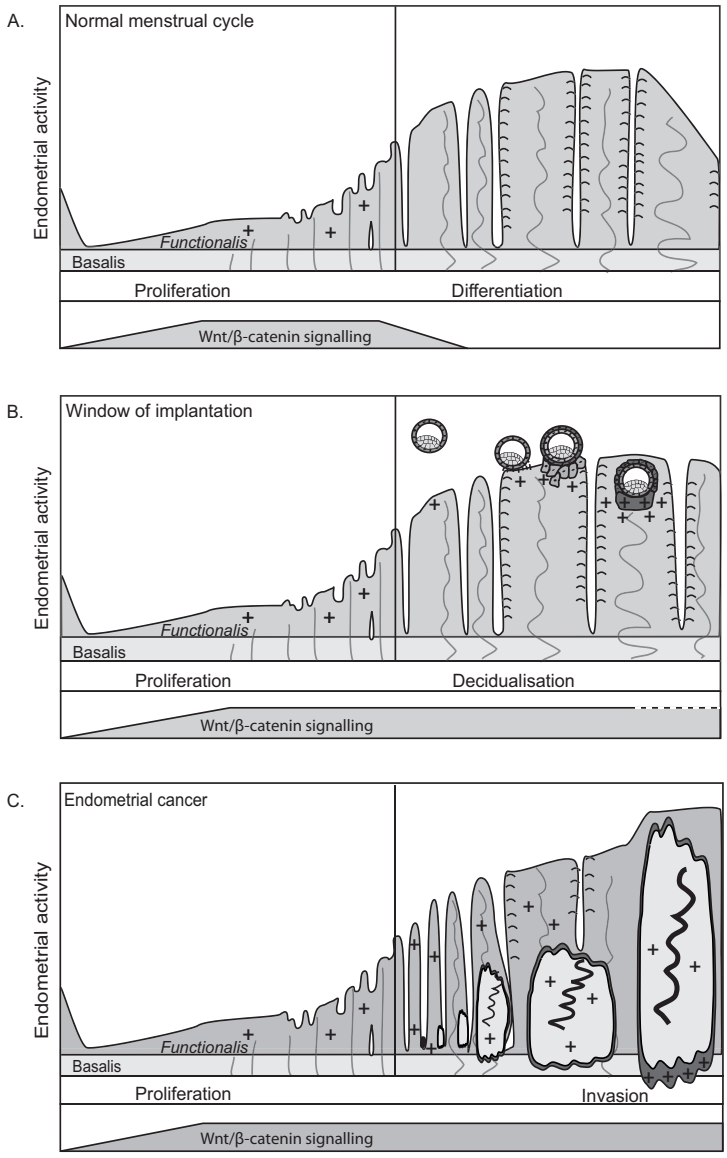
Nei et al. in 1999 observed clear nuclear localization of β-catenin during the proliferative phase of the menstrual cycle when estrogen levels are high and unopposed by progestagens[20]. Furthermore, during the secretory phase of the menstrual cycle, when progesterone levels increase and estrogen levels decrease, nuclear β-catenin accumulation was found to decrease. In line with these observations Hou et al., 2004, showed that exogenous estrogen treatment of mice indeed results in nuclear localization of β-catenin in epithelial cells of the endometrium[21]. They also observed that the proliferative effect of estrogens could be inhibited by adenovirus mediated in vivo uterine delivery of *Sfrp2* (a known Wnt antagonist)[21]. In agreement with these observations, Gunin et al., 2004 could mimic estrogens proliferative effects on the endometrium by feeding their mice LiCl, which is known to activate Wnt/β-catenin signalling by inhibiting Gsk3b activity[22].

More data suggesting involvement of Wnt/β-catenin signalling in regulation of the menstrual cycle came from gene expression profiling studies[23-27]. Wang et al., 2009[28] combined two large sets of endometrial gene expression data: gene expression profiles from normal human endometrial tissue acquired during different phases of the menstrual cycle[27], and endometrial gene expression data from postmenopausal women that were either untreated or were treated with estrogen or estrogen+progestagen[25]. Combining these two data sets, large numbers of differentially expressed genes were recognized as either downstream targets or integral parts of the Wnt/β-catenin signalling pathway (n=228,[28]). For example, *WNT4, WNT5a, WNT6* and *WNT7a* were up regulated by estrogen during the proliferative phase of the menstrual cycle, while a number of inhibitors of Wnt/β-catenin signalling were found up regulated by progesterone during the secretory phase of the menstrual cycle (the complete list of regulated genes can be accessed from Supplementary Table 1).

DKK1 and FOXO1 are two progesterone regulated Wnt/β-catenin signalling inhibitors which have been investigated further[28]. Progesterone regulation of DKK1 was first observed by Kao et al., 2002,[29] and Tulac et al., 2003,[30, 31] in stromal cells of the human endometrium and progesterone regulation of FOXO1 has been extensively studied by Takano et al., 2007, and Ward et al., 2008[32, 33]. Using the human endometrial cancer cell line Ishikawa Wang et al., 2009,[28] has investigated progesterone inhibition of Wnt/β-catenin signalling and the involvement of DKK1 and FOXO1 further. Here it was shown that progesterone was very effective in inhibiting the TOP-Flash Wnt/β-catenin signalling reporter in Ishikawa cells[28]. Furthermore, when progesterone was added to the medium and DKK1 or FOXO1 expression was inhibited by use of specific siRNAs, progesterone inhibition of Wnt/β-catenin signalling was partly circumvented indicating that the Wnt/β-catenin signalling inhibitors DKK1 and FOXO1 acted downstream from progesterone.

In summary, sexhormones regulate Wnt/β-catenin signalling in the endometrium to maintain the monthly balance between estrogen-induced proliferation and progesterone-induced differentiation (Table 1 and Fig. 1).

**Figure 1: Activation of Wnt/β-catenin signalling during the normal menstrual cycle (A), the window of implantation (B) and endometrial carcinogenesis (C).**




A: During the proliferative phase of the menstrual cycle, estrogens induce Wnt/β-catenin signalling, while during the secretory phase of the menstrual cycle progestagens counterbalance estrogen-induced proliferation by inhibition of Wnt/β-catenin signalling, thus inducing differentiation. B: During implantation, blastocyst signalling to the endometrium activates the Wnt/β-catenin pathway at the site of implantation. Furthermore activation of Wnt/β-catenin signalling is a prerequisite for proper decidualization and correct invasion of trophoblast into the maternal endometrium. C: Constitutive activation of Wnt/β-catenin signalling in the endometrium induces endometrial hyperplasia, which can develop further into invasive disease. Furthermore, once a tumour has been initiated, Wnt/β-catenin signalling seems to facilitate transition from an epithelial phenotype towards a mesenchymal phenotype thus aiding endometrial cancer progression. **+**: represents locations where Wnt/β-catenin signalling is activated. *Figure modified from Wang et al., Oncotarget 2010.*

## Wnt/β-catenin signalling during decidualization, implantation and placenta formation:

In humans, fertilization occurs within 24 to 48 hours after ovulation when the oocyte travels through the fallopian tube towards the uterine cavity. When the embryo reaches the uterus it has developed into a fluid filled mass of cells (blastocyst) displaying the first signs of differentiation. Within 72 hours of reaching the uterus, the blastocyst is released from the surrounding zona pellucida (hatching) thus exposing its surface cells (trophoblast) to the endometrial epithelium. The first step towards implantation involves adhesion of these trophoblasts to the uterine wall (apposition), which is followed by stabilization of binding (stable attachment). Subsequently, invasion begins by penetration of the syncytiotrophoblasts into uterine epithelium[34, 35]. Ten days after conception, the embryo has completely invaded into the endometrium and mononuclear cytotrophoblasts start to invade the endometrium and inner third of the myometrium.

Receptivity of the endometrium for implantation depends highly on correct hormonal signalling towards the moment of implantation (between days 20 and 24 of the menstrual cycle). Estrogens, produced in increasing amounts during the first two weeks of the menstrual cycle, induce outgrowth of the functional layer of the endometrium. Progesterone, being produced from the moment of ovulation onwards, is very effective in inhibiting estrogenic effects and induces differentiation. Differentiation is characterized by induction of secretory activity of the glands, attraction of natural killer cells and initiation of transformation of endometrial stroma cells into decidual cells (start of decidualization)[36]. This endometrial priming in humans is, in contrast to the situation in mice where decidualization starts after implantation, a crucial step towards implantation, invasion of trophoblasts and full decidualization of the uterine stroma[37].

Based on the fact that Wnt/β-catenin signalling plays an important role in proliferation and differentiation during normal uterine physiology and that Wnt/β-catenin signalling has an essential function in embryonic development, a role for Wnt/β-catenin signalling in blastocyst implantation, endometrial decidualization and placenta formation was hypothesized (recently reviewed by Sonderegger et al., 2010[38]).

In wild-type mice, Hayashi et al., 2009, studied the expression of different Wnt receptors (*Fzd2, Fzd3* and *Fzd4*) and ligands (*Wnt4, Wnt5a, Wnt7a, Wnt7b, Wnt11, Wnt16*) during peri-implantation of pregnancy and it was observed that, except for Fzd6, all receptors and ligands were specifically expressed at the site of implantation and around the moment of implantation[39]. Furthermore, expression of *Wnt4, Wnt7a, Wnt7b, Wnt11, Wnt16, Fzd2, Fzd4* and *Fzd6* was found to be regulated in ovariectomized mice treated with estradiol and/or progesterone[39]. Using *Tcf/Lef-LacZ* reporter mice, Mohamed et al., 2005, actually measured activation of the Wnt/β-catenin signalling pathway during the window of implantation[40]. It was observed that 4 days after fertilization, 5 – 7 bands of transient Wnt/β-catenin activity were present in the inner circular smooth muscle layer of the myometrium, probably marking future sites of implantation. Subsequently, at day 5 after fertilization, the Wnt/β-catenin signalling pathway was activated in specific endometrial regions in the vicinity of a blastocyst, indicating cross-talk between the blastocyst and the endometrium[40].

Furthermore, instead of assessing pregnant mice, pseudopregnant mice were injected with Wnt7a and profound activation of Wnt/β-catenin signalling was observed throughout the exposed region. Next, the authors showed that when mice blastocysts were treated with the Wnt/β-catenin signalling inhibitor Sfrp2 or when high amounts of Sfrp2 were present during implantation, the implantation rate dropped by approximately 50%[40]. In addition, Xie et al., 2008, inhibited Wnt/β-catenin signalling in mice blastocysts using adenoviral delivered Dkk1 and also observed profound inhibition of implantation in normal pseudopregnant recipients[41]. These investigations indicate that in mice, embryo-induced Wnt/β-catenin signalling at the site of blastocyst attachment is prerequisite for successful implantation[40-42].

During implantation stromal cells of the endometrium undergo further decidualization. Interestingly in humans and in pregnant mice, during the secretory phase of the menstrual cycle, progesterone induced Wnt4 expression was shown to be responsible for Bmp2 mediated decidualization[43, 44]. Wnt4 acts downstream from Bmp2 and *Wnt4* conditional knockdown in mice was shown to affect stromal cell survival, differentiation and responsiveness to progestagens[45]. Furthermore, Cloke et al., 2008, indicated that next to progesterone signalling also androgen signalling was involved in decidualization although androgen action does not seem to be mediated by the Wnt/β-catenin signalling pathway[24].

During implantation, placental formation is initiated as trophoblast cells start to invade into the underlying decidualized maternal tissue. Subsequently, maternal blood vessels are broken down by these invading trophoblasts, thus forming blood sinuses. In mice, these blood sinuses are invaded by foetal vessels and capillaries (produced from the allantois) establishing the so called labyrinthine zone[46]. A number of genetic mouse models support the hypothesis that Wnt/β-catenin signalling activation is an important factor allowing trophoblast migration, placental vascularisation, chorion allantois fusion and labyrinth function thus initiating a functional placenta. In mice, Wnt2 has been shown to be expressed on the foetal side of the developing placenta and targeted disruption of *Wnt2,* interestingly, resulted in placental defects caused by improper and defective vascularisation of the placenta[47]. In addition, Wnt7b is expressed in the chorion and disruption of this gene in mice results in embryonic death at midgestation. More in detail, chorion development was found to be impaired as a consequence of absence of fusion (decreased cell adhesion through down regulation of Wnt/β-catenin signalling target gene α4-integrin) between the allantois and chorion, possibly causing a severe lack of nutrient supply from the mother[48]. Targeted disruption of *Tcf1* or *Lef1*, interestingly, also resulted in defects in the formation of the placenta due to loss of allantois-chorion fusion[49]. Furthermore Fzd5 was found to be important for placenta development, as *Fzd5* knock-out mice died in utero displaying poor placental vascularisation[50].

In humans, many Wnt ligands and FZD receptors are detectable in placental tissues[51] and recent studies have indicated increased expression of TCF3/4 and nuclear β-catenin staining in invasive trophoblasts during the early phases of placentation[52]. Furthermore, recombinant Wnt-3A treatment of human trophoblasts induced the activity of the Wnt/β-catenin reporter *TCF-luciferase*, and was shown to induce secretion of MMP2, which could help promote trophoblast migration and invasion[53]. In agreement with this, treatment of primary human trophoblasts with the Wnt/β-

catenin inhibitor DKK1 resulted in reduced migration and invasion[52]. Recently, gene expression was studied in human embryonic stem cells that were differentiated down the trophoblast lineage by culture with BMP4, and profound regulation of the Wnt/β-catenin pathway was observed[54].

The involvement of Wnt/β-catenin signalling in migration and invasion is not a new finding. A role for β-catenin-independent Wnt signalling in migration and invasion has also been described for gliomas[55] and breast cancer metastasis in the brain[56]. For β-catenin-dependent Wnt signalling Schmalhofer et al., 2009, showed clear nuclear β-catenin staining at the invasive front of progressive colorectal cancer, further indicating a role for Wnt/β-catenin signalling in epithelial to mesenchymal transformation[57]. In addition, in endometrial cancer the Wnt/β-catenin signalling pathway target and adhesion molecule L1CAM was also shown to be present specifically at the leading edge of the tumour[58].

In summary, blastocyst signalling to the endometrium activates the Wnt/β-catenin signalling pathway at the site of implantation and is prerequisite for proper implantation. Activation of the Wnt/β-catenin signalling pathway, furthermore, is a requirement for proper decidualization and correct invasion of trophoblasts into the maternal endometrium and myometrium thus forming the placenta (Table 1 and Fig. 1).

## Wnt/β-catenin signalling in endometriosis:

Endometriosis, a common and benign gynaecological disorder, is characterised by the presence of endometrial glandular and stromal tissue outside the uterine cavity (pelvic peritoneum, on the ovaries and in the rectovaginal septum) and is associated with pelvic pain and infertility. Because endometriosis is an estrogen-dependent disease displaying reduced progesterone receptor levels and resistance to progesterone therapy[59-62], a role for Wnt/β-catenin signalling in development and maintenance of the disease has been proposed.

Using gene expression profiling, indications were found that Wnt/β-catenin signalling was indeed differentially regulated between eutopic and ectopic endometrium[60, 63, 64]. Furthermore, Gaetje et al. in 2007, showed significantly higher expression of WNT7a in endometriotic tissues, most likely caused by reduced progesterone signalling[65]. This is an interesting finding because WNT7A has been described to induce HOXA10 expression which is strongly implicated in the development of endometriosis[66].

Besides endometrial tissues homing towards the abdominal cavity, there is a special form of endometriosis which invades into the myometrium called adenomyosis. Interestingly, using a mouse model where Wnt/β-catenin signalling was activated in the myometrium, endometrial glands and stroma were observed to be present in the myometrium[67, 68]. Whether these observations point towards an active process of endometrial tissue invading into the myometrium or perhaps endometrial tissue is simply filling the gap generated by myometrial dystrophy, is not entirely clear at this point.

In summary, enhanced estrogen signalling relative to inhibited progesterone signalling in ectopic endometrium activates the Wnt/β-catenin signalling pathway, and may be a mechanism stimulating survival, proliferation and invasion of endometrial tissue outside its normal environment (Table 1).

## Wnt/β-catenin signalling during endometrial carcinogenesis:

Major risk factors for endometrial cancer are prolonged high levels of estrogens[69]. During the normal menstrual cycle, high estrogen levels are counterbalanced each month by progesterone during the secretory phase of the menstrual cycle. When these progesterone levels are too low, or when estrogen levels are too high, the proliferative effect of estrogen becomes dominant and will induce endometrial hyperplasia[70]. Endometrial hyperplasia can, over time, develop further into type I endometrial cancer which makes up 90% of endometrial cancer cases[70].

As indicated earlier, estrogens seem to induce Wnt/β-catenin signalling during the proliferative phase of the menstrual cycle[20] and artificial induction of Wnt/β-catenin signalling results in endometrial hyperplasia[22, 71]. Based on these investigations, it was hypothesized that enhanced Wnt/β-catenin signalling could be a causative factor in endometrial hyperplasia and in endometrial carcinogenesis. In agreement with this Wnt/β-catenin signalling, as measured by nuclear β-catenin staining, was found to be enhanced in about 40% of well differentiated endometrial cancers (31%:[72]; 85%:[73]). Upon investigating the mechanism behind enhanced nuclear β-catenin staining, activating β-catenin mutations were found in 15-40 % of endometrial tumours[74, 75], truncating *APC* mutations in 10% of all endometrial cancers[76] and *APC* A1 promoter hypermethylation in approximately 20% of endometrial cancers[77]. These findings seem to indicate that Wnt/β-catenin signalling plays a significant role during endometrial carcinogenesis.

Using genetically modified mice the role of Wnt/β-catenin signalling during endometrial carcinogenesis was investigated further. Because homozygous β-catenin deletion results in embryo lethality, conditional knockdown was established using β-catenin gene targeting with the help of C-recombinase, Cre[78]. Using this technique the β-catenin gene (*Ctnnb1*) is knocked out in a specific tissue at a specific time. In *Amhr2-Cre* mice, Cre is expressed from E-12.5 onwards in mesenchymal cells surrounding the Müllerian duct[79, 80]. In adult animals *Amhr2* driven Cre-expression was clearly observed in the myometrium but expression was much lower in endometrial stroma cells and Cre was not expressed in epithelial cells[79, 81]. At birth, in β-catenin conditional knockdown animals a smaller uterus was observed (due to decreased mesenchymal and epithelial cell proliferation) and coiling of the oviduct was sometimes impaired (resembling the *Wnt7a* mutant [14])[81, 82]. Interestingly in adult animals, over time myometrial cells were lost (dystrophy, resembling the *Wnt7a* mutant[14]) and vast areas of adiposites appeared. This phenotype seems, to some extent, to resemble a human condition called lipoleiomyoma[82].

Tanwar et al., 2009, used *Amhr2-Cre* to induce an activating mutation of β-catenin and macroscopically found large tumourous growths and multiple hemorrhagic sites on the uterine surface[67]. Microscopically the authors observed an increase in the myometrial area and TGFβ3 positive dysplastic lesions of the myometrium (resembling human uterine leiomyomas). In addition, endometrial stromal sarcomas and epithelial hyperplasia were observed. Finally, endometrial glands were sometimes observed inside the myometrium, resembling a human situation called adenomyosis (as discussed before). Recently, Tanwar et al., 2011, used *Amhr2-Cre* to force *Apc* deletion to induce Wnt/β-catenin signalling. It was observed that besides myometrial defects these animals displayed endometrial hyperplasia and cancer combined with defective estrogen signalling[83].

**Table 1: Summary of WNT/β-catenin signalling in endometrial physiology and disease.**

| | | |
|---|---|---|
| **Wnt/β-catenin signalling in Müllerian duct development:** | | |
| Wnt4 | - Wnt4 is expressed by epithelial cells from the mesonephros or coelomic wall | [9-10] |
| | - Wnt4 is a prerequisite for Müllerian duct initiation | [9-10, 15-16] |
| Wnt5a | - Wnt5a is expressed in mesenchymal cells of the Müllerian duct | [18] |
| | - Wnt5a knockout mice lack cervical and vaginal structures | [13] |
| Wnt7a | - Wnt7a is expressed throughout the Müllerian duct epithelium | [14] |
| | - Wnt7a loss results in posteriorization of the female reproductive tract | [14,17] |
| Wnt9a | - Wnt9b is expressed in epithelial cells from the Wolffian duct, when the Müllerian duct is present | [10] |
| | - Wnt9b is a prerequisite for posterior outgrowth of the early Müllerian duct | [10] |
| **Wnt/β-catenin signalling in uterine physiology:** | | |
| DKK1 | - DKK1 is progesterone induced and can inhibit Wnt/β-catenin signalling | [28-31] |
| FOXO1 | - FOXO1 is progesterone induced and can inhibit Wnt/β-catenin signalling | [28, 32-33] |
| Gsk3b | - Gsk3b inhibition leads to Wnt signaling activation and mimics estrogens induced proliferation | [22] |
| Sfrp2 | - Sfrp2, a known Wnt antagonist, opposes the proliferative effect of estrogen | [20] |
| **Wnt/β-catenin signalling during decidualization, implantation and placenta formation** | | |
| Dkk1 | - Dkk1 treatment inhibits implantation in normal pseudopregnant recipients | [41] |
| DKK1 | - DKK1 treatment results in reduced trophoblast migration and invasion | [52] |
| Fzd5 | - Fzd5 knockout results in embryonic death through poor placental vascularisation | [50] |
| Lef1 | - Lef1 targeted disruption results in defects in placental formation | [49] |
| Sfrp2 | - Sfrp2 treatment inhibits implantation in mice | [40] |
| Tcf1 | - Tcf1 targeted disruption results in defects in placental formation | [49] |
| Wnt2 | - Wnt2 targeted disruption results in defective placental vascularisation | [47] |
| Wnt3a | - Wnt3a treatment promotes trophoblast migration and invasion | [53] |
| Wnt4 | - Wnt4 is responsible for Bmp2 mediated decidualisation | [43-44] |
| | - Wnt4 knockout affects stromal cell survival, differentiation and progesterone responsiveness | [45] |
| Wnt7a | - Wnt7a activates Wnt/β-catenin signalling in pseudopregnant mice | [40] |
| Wnt7b | - Wnt7b disruption results in embryonic death due to placental failure | [48] |
| **Wnt/β-catenin signalling in endometriosis and endometrial carcinogenesis:** | | |
| Apc | - Apc conditional knockdown results in endometrial hyperplasia and cancer | [82-83] |
| | - Apc conditional knockdown results in myometrial loss and reduced gland numbers | [68] |
| APC | - APC is mutated in 10% and its promoter hypermethylated in 20% of endometrial cancers | [75-76] |
| β-catenin | - Activating β-catenin mutations were found in 15-40 % of endometrial cancers | [73-74] |
| | - Conditional activation of β-catenin in mice results in tumour-like growths and multiple hemorrhagic sites at the uterine surface; increased myometrial area and TGFβ3 positive dysplastic lesions of the myometrium; endometrial stromal sarcomas; enlarged glands causing epithelial hyperplasia and sometimes endometrial glands were observed inside the myometrium. | [67] |
| | - Conditional knockdown of β-catenin results in myometrial loss and areas of adipogenesis; less epithelial glands and squamous cell metaplasia. | [81] |
| WNT7A | - WNT7A is enhanced in endometriosis and induces HOXA10 | [65-66] |

Not included in this summary are studies using gene expression analysis (micro-array and RT-PCR) that show WNT/β-catenin signalling involvement [23-28, 39, 54, 60, 63-64].

Our own data, using *Amhr2-Cre* to drive *Apc* deletion, also indicate severe myometrial defects and reduced endometrial gland formation as a result of induction of Wnt/β-catenin signalling in mesenchymal cells surrounding the Müllerian duct[68]. However, in these animals we never observed endometrial hyperplasia nor endometrial carcinogenesis.

Jeong et al., 2009, used the progesterone receptor to drive Cre expression in order to induce an activating or inactivating mutation of β-catenin in all uterine cells (myometrium, stroma, glandular epithelium and luminal epithelium)[84]. Both β-catenin mutations led to severe subfertility or even infertility due to failure to undergo decidualization during embryo implantation. Furthermore, *Pgr-Cre* induced constitutive β-catenin activation resulted in enlarged glands causing endometrial hyperplasia. Conditional inactivation of β-catenin, however, resulted in less epithelial glands and squamous cell metaplasia (resembling the *Wnt7a* mutant[17]). Recently our group has also used *Pgr-Cre* to drive deletion of *Apc* and we observed clear endometrial hyperplasia which was sporadically followed in time by endometrial carcinogenesis.

Recently we have been investigating progressive endometrial cancer and observed that loss of progesterone signalling seems to release inhibition of epithelial to mesenchymal cell transition thus facilitating tumour progression and malignant transformation. Interestingly, loss of progesterone signalling also led to enhanced Wnt/β-catenin signalling in these progressive endometrial cancer specimens (Van der Horst et al., submitted).

In summary, enhanced Wnt/β-catenin signalling in mesenchymal cells surrounding the Müllerian duct results in severe myometrial problems, while continuous Wnt/β-catenin signalling in the endometrium seems to be an important early step in endometrial carcinogenesis (Table 1 and Fig. 1).

## Summary:

The role of Wnt signalling in initiation, development and function of the female reproductive tract is significant. During development, Wnt4 is essential for initiation of the Müllerian duct, Wnt9b is essential for posterior outgrowth of the Müllerian duct and Wnt5a and Wnt7a are involved in proper differentiation of the Müllerian duct, into vagina, cervix, uterus and oviduct[10, 13, 14, 16]. During reproductive life, hormonal regulation of the menstrual cycle is mediated by estrogen induced activation and progesterone induced inhibition of Wnt/β-catenin signalling[20, 21, 23, 25, 28, 30]. During pregnancy, when the embryo is nearing its site of implantation, Wnt/β-catenin signalling is profoundly induced and during early decidualization, Bmp2 induced Wnt4 signalling allows for stromal survival and differentiation. Next to this, genetic models showed that Wnt2 and Wnt7b are essential for invasion of trophoblasts that can form the interphase where exchange can take place between mother and foetus (the placenta)[40, 44, 48].

These normal functions of Wnt/β-catenin signalling, however, have a down site. Constitutively activated Wnt/β-catenin signalling in the myometrial layer of the uterus can cause muscular dystrophy, probably facilitating placental invasion during pregnancy. At the same time Wnt/β-catenin signalling in the myometrium seems to induce a disorder called adenomyosis[67, 68, 81, 82].

Furthermore, activated Wnt/β-catenin signalling is involved in estrogen induced proliferation of the endometrium during the first two weeks of the menstrual cycle. However, constitutive Wnt signalling in the endometrium induces endometrial hyperplasia which may proceed to endometrial cancer. Also, similar to Wnt/β-catenin activation during trophoblast invasion, once a tumour has been initiated constitutive Wnt/β-catenin signalling seems to facilitate transition from an epithelial phenotype towards a mesenchymal phenotype thus aiding endometrial cancer progression[22, 71, 74-77].

# References


1. Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 1982;**31:**99-109.
2. Clevers H. Wnt/[beta]-Catenin Signaling in Development and Disease. *Cell* 2006;**127:**469-80.
3. Katoh M. WNT/PCP signaling pathway and human cancer (review). *Oncol Rep* 2005;**14:**1583-8.
4. Kohn AD, Moon RT. Wnt and calcium signaling: beta-catenin-independent pathways. *Cell Calcium* 2005;**38:**439-46.
5. Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 2000;**407:**535-8.
6. Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, et al. Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. *Science* 1998;**280:**596-9.
7. Daniels DL, Weis WI. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat Struct Mol Biol* 2005;**12:**364-71.
8. Nusse R. The Wnt Homepage http://www.stanford.edu/group/nusselab/cgi-bin/wnt/.
9. Kobayashi A, Shawlot W, Kania A, Behringer RR. Requirement of Lim1 for female reproductive tract development. *Development* 2004;**131:**539-49.
10. Carroll TJ, Park JS, Hayashi S, Majumdar A, McMahon AP. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev Cell* 2005;**9:**283-92.
11. Guioli S, Sekido R, Lovell-Badge R. The origin of the Mullerian duct in chick and mouse. *Dev Biol* 2007;**302:**389-98.
12. Orvis GD, Behringer RR. Cellular mechanisms of Mullerian duct formation in the mouse. *Dev Biol* 2007;**306:**493-504.
13. Mericskay M, Kitajewski J, Sassoon D. Wnt5a is required for proper epithelial-mesenchymal interactions in the uterus. *Development* 2004;**131:**2061-72.
14. Miller C, Sassoon DA. Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. *Development* 1998;**125:**3201-11.
15. Stark K, Vainio S, Vassileva G, McMahon AP. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature* 1994;**372:**679-83.
16. Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP. Female development in mammals is regulated by Wnt-4 signalling. *Nature* 1999;**397:**405-9.
17. Parr BA, McMahon AP. Sexually dimorphic development of the mammalian reproductive tract requires Wnt-7a. *Nature* 1998;**395:**707-10.
18. Miller C, Pavlova A, Sassoon DA. Differential expression patterns of Wnt genes in the murine female reproductive tract during development and the estrous cycle. *Mech Dev* 1998;**76:**91-9.
19. Maruyama T, Yoshimura Y. Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium. *Endocr J* 2008;**55:**795-810.
20. Nei H, Saito T, Yamasaki H, Mizumoto H, Ito E, et al. Nuclear localization of β-catenin in normal and carcinogenic endometrium. *Molecular Carcinogenesis* 1999;**25:**207-18.
21. Hou X, Tan Y, Li M, Dey SK, Das SK. Canonical Wnt signaling is critical to estrogen-mediated uterine growth. *Mol Endocrinol* 2004;**18:**3035-49.
22. Gunin AG, Emelianov VU, Mironkin IU, Morozov MP, Tolmachev AS. Lithium treatment enhances estradiol-induced proliferation and hyperplasia formation in the uterus of mice. *Eur J Obstet Gynecol Reprod Biol* 2004;**114:**83-91.
23. Catalano RD, Critchley HO, Heikinheimo O, Baird DT, Hapangama D, et al. Mifepristone induced progesterone withdrawal reveals novel regulatory pathways in human endometrium. *Molecular Human Reproduction* 2007;**13:**641-54.
24. Cloke B, Huhtinen K, Fusi L, Kajihara T, Yliheikkila M, et al. The Androgen and Progesterone Receptors Regulate Distinct Gene Networks and Cellular Functions in Decidualizing Endometrium. *Endocrinology* 2008;**149:**4462-74.
25. Hanifi-Moghaddam P, Boers-Sijmons B, Klaassens AH, van Wijk FH, den Bakker MA, et al. Molecular analysis of human endometrium: short-term tibolone signaling differs significantly from estrogen and estrogen + progestagen signaling. *J Mol Med* 2007;**85:**471-80.

26. Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N, et al. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. *Biol Reprod* 2010;**82:**791-801.
27. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* 2006;**147:**1097-121.
28. Wang Y, Hanifi-Moghaddam P, Hanekamp EE, Kloosterboer HJ, Franken P, et al. Progesterone inhibition of Wnt/beta-catenin signaling in normal endometrium and endometrial cancer. *Clin Cancer Res* 2009;**15:**5784-93.
29. Kao LC, Tulac S, Lobo S, Imani B, Yang JP, et al. Global Gene Profiling in Human Endometrium during the Window of Implantation. *Endocrinology* 2002;**143:**2119-38.
30. Tulac S, Nayak NR, Kao LC, Van Waes M, Huang J, et al. Identification, characterization, and regulation of the canonical Wnt signaling pathway in human endometrium. *J Clin Endocrinol Metab* 2003;**88:**3860-6.
31. Tulac S, Overgaard MT, Hamilton AE, Jumbe NL, Suchanek E, et al. Dickkopf-1, an Inhibitor of Wnt Signaling, Is Regulated by Progesterone in Human Endometrial Stromal Cells. *J Clin Endocrinol Metab* 2006;**91:**1453-61.
32. Takano M, Lu Z, Goto T, Fusi L, Higham J, et al. Transcriptional Cross Talk between the Forkhead Transcription Factor Forkhead Box O1A and the Progesterone Receptor Coordinates Cell Cycle Regulation and Differentiation in Human Endometrial Stromal Cells. *Mol Endocrinol* 2007;**21:**2334-49.
33. Ward EC, Hoekstra AV, Blok LJ, Hanifi-Moghaddam P, Lurain JR, et al. The regulation and function of the forkhead transcription factor, Forkhead box O1, is dependent on the progesterone receptor in endometrial carcinoma. *Endocrinology* 2008;**149:**1942-50.
34. Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, et al. Embryo implantation. *Dev Biol* 2000;**223:**217-37.
35. Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. *N Engl J Med* 2001;**345:**1400-8.
36. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. *Semin Reprod Med* 2007;**25:**445-53.
37. Dey SK, Lim H, Das SK, Reese J, Paria BC, et al. Molecular cues to implantation. *Endocr Rev* 2004;**25:**341-73.
38. Sonderegger S, Pollheimer J, Knöfler M. Wnt Signalling in Implantation, Decidualisation and Placental Differentiation - Review. *Placenta* 2010;**31:**839-47.
39. Hayashi K, Erikson DW, Tilford SA, Bany BM, Maclean JA, et al. Wnt Genes in the Mouse Uterus: Potential Regulation of Implantation. *Biology of Reproduction* 2009;**80:**989-1000.
40. Mohamed OA, Jonnaert M, Labelle-Dumais C, Kuroda K, Clarke HJ, et al. Uterine Wnt/beta-catenin signaling is required for implantation. *Proc Natl Acad Sci U S A* 2005;**102:**8579-84.
41. Xie H, Tranguch S, Jia X, Zhang H, Das SK, et al. Inactivation of nuclear Wnt-beta-catenin signaling limits blastocyst competency for implantation. *Development* 2008;**135:**717-27.
42. Mohamed OA, Dufort D, Clarke HJ. Expression and Estradiol Regulation of Wnt Genes in the Mouse Blastocyst Identify a Candidate Pathway for Embryo-Maternal Signaling at Implantation. *Biology of Reproduction* 2004;**71:**417-24.
43. Lee KY, Jeong JW, Wang J, Ma L, Martin JF, et al. Bmp2 is critical for the murine uterine decidual response. *Mol Cell Biol* 2007;**27:**5468-78.
44. Li Q, Kannan A, Wang W, DeMayo FJ, Taylor RN, et al. Bone Morphogenetic Protein 2 Functions via a Conserved Signaling Pathway Involving Wnt4 to Regulate Uterine Decidualization in the Mouse and the Human. *Journal of Biological Chemistry* 2007;**282:**31725-32.
45. Franco HL, Dai D, Lee KY, Rubel CA, Roop D, et al. WNT4 is a key regulator of normal postnatal uterine development and progesterone signaling during embryo implantation and decidualization in the mouse. *FASEB J* 2010.
46. Torry DS, Leavenworth J, Chang M, Maheshwari V, Groesch K, et al. Angiogenesis in implantation. *J Assist Reprod Genet* 2007;**24:**303-15.
47. Monkley SJ, Delaney SJ, Pennisi DJ, Christiansen JH, Wainwright BJ. Targeted disruption of the Wnt2 gene results in placentation defects. *Development* 1996;**122:**3343-53.
48. Parr BA, Cornish VA, Cybulsky MI, McMahon AP. Wnt7b regulates placental development in mice. *Dev Biol* 2001;**237:**324-32.

49. Galceran J, Farinas I, Depew MJ, Clevers H, Grosschedl R. Wnt3a-/--like phenotype and limb deficiency in Lef1(-/-)Tcf1(-/-) mice. *Genes Dev* 1999;**13:**709-17.
50. Ishikawa T, Tamai Y, Zorn AM, Yoshida H, Seldin MF, et al. Mouse Wnt receptor gene Fzd5 is essential for yolk sac and placental angiogenesis. *Development* 2001;**128:**25-33.
51. Sonderegger S, Husslein H, Leisser C, Knöfler M. Complex Expression Pattern of Wnt Ligands and Frizzled Receptors in Human Placenta and its Trophoblast Subtypes. *Placenta* 2007;**28:**S97-S102.
52. Pollheimer J, Loregger T, Sonderegger S, Saleh L, Bauer S, et al. Activation of the canonical wingless/T-cell factor signaling pathway promotes invasive differentiation of human trophoblast. *Am J Pathol* 2006;**168:**1134-47.
53. Sonderegger S, Haslinger P, Sabri A, Leisser C, Otten JV, et al. Wingless (Wnt)-3A induces trophoblast migration and matrix metalloproteinase-2 secretion through canonical Wnt signaling and protein kinase B/AKT activation. *Endocrinology* 2010;**151:**211-20.
54. Marchand M, Horcajadas JA, Esteban FJ, McElroy SL, Fisher SJ, et al. Transcriptomic Signature of Trophoblast Differentiation in a Human Embryonic Stem Cell Model. *Biol Reprod* 2011.
55. Kamino M, Kishida M, Kibe T, Ikoma K, Iijima M, et al. Wnt-5a signaling is correlated with infiltrative activity in human glioma by inducing cellular migration and MMP-2. *Cancer Sci* 2011;**102:**540-8.
56. Klemm F, Bleckmann A, Siam L, Chuang HN, Rietkotter E, et al. {beta}-catenin-independent WNT signaling in basal-like breast cancer and brain metastasis. *Carcinogenesis* 2011;**32:**434-42.
57. Schmalhofer O, Brabletz S, Brabletz T. E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. *Cancer Metastasis Rev* 2009;**28:**151-66.
58. Huszar M, Pfeifer M, Schirmer U, Kiefel H, Konecny GE, et al. Up-regulation of L1CAM is linked to loss of hormone receptors and E-cadherin in aggressive subtypes of endometrial carcinomas. *J Pathol* 2010;**220:**551-61.
59. Giudice LC, Kao LC. Endometriosis. *Lancet* 2004;**364:**1789-99.
60. Burney RO, Talbi S, Hamilton AE, Vo KC, Nyegaard M, et al. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. *Endocrinology* 2007;**148:**3814-26.
61. Bulun SE, Cheng YH, Pavone ME, Xue Q, Attar E, et al. Estrogen receptor-beta, estrogen receptor-alpha, and progesterone resistance in endometriosis. *Semin Reprod Med* 2010;**28:**36-43.
62. Bulun SE, Cheng YH, Pavone ME, Yin P, Imir G, et al. 17Beta-hydroxysteroid dehydrogenase-2 deficiency and progesterone resistance in endometriosis. *Semin Reprod Med* 2010;**28:**44-50.
63. Kao LC, Germeyer A, Tulac S, Lobo S, Yang JP, et al. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology* 2003;**144:**2870-81.
64. Wu Y, Kajdacsy-Balla A, Strawn E, Basir Z, Halverson G, et al. Transcriptional Characterizations of Differences between Eutopic and Ectopic Endometrium. *Endocrinology* 2006;**147:**232-46.
65. Gaetje R, Holtrich U, Karn T, Cikrit E, Engels K, et al. Characterization of WNT7A expression in human endometrium and endometriotic lesions. *Fertility and Sterility* 2007;**88:**1534-40.
66. Zanatta A, Rocha AM, Carvalho FM, Pereira RM, Taylor HS, et al. The role of the Hoxa10/HOXA10 gene in the etiology of endometriosis and its related infertility: a review. *J Assist Reprod Genet* 2010;**27:**701-10.
67. Tanwar PS, Lee H-J, Zhang L, Zukerberg LR, Taketo MM, et al. Constitutive Activation of Beta-Catenin in Uterine Stroma and Smooth Muscle Leads to the Development of Mesenchymal Tumors in Mice. *Biology of Reproduction* 2009;**81:**545-52.
68. Wang Y, Jia Y, Franken PF, Smits MJM, Ewing PC, et al. Loss of APC function in mesenchymal cells surrounding the Müllerian duct leads to myometrial defects in adult mice. *Mol Cell Endocrinol* Accepted 2011.
69. Schindler AE. Progestogen deficiency and endometrial cancer risk. *Maturitas* 2009;**62:**334-7.
70. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, et al. Endometrial cancer. *Lancet* 2005;**366:**491-505.
71. Polotsky AJ, Zhu L, Santoro N, Pollard JW. Lithium chloride treatment induces epithelial cell proliferation in xenografted human endometrium. *Hum Reprod* 2009;**24:**1960-7.
72. Scholten AN, Creutzberg CL, van den Broek LJ, Noordijk EM, Smit VT. Nuclear beta-catenin is a molecular feature of type I endometrial carcinoma. *J Pathol* 2003;**201:**460-5.

73. Saegusa M, Okayasu I. Frequent nuclear beta-catenin accumulation and associated mutations in endometrioid-type endometrial and ovarian carcinomas with squamous differentiation. *J Pathol* 2001;**194:**59-67.

74. Hecht JL, Mutter GL. Molecular and pathologic aspects of endometrial carcinogenesis. *J Clin Oncol* 2006;**24:**4783-91.

75. Konopka B, Janiec-Jankowska A, Czapczak D, Paszko Z, Bidzinski M, et al. Molecular genetic defects in endometrial carcinomas: microsatellite instability, PTEN and beta-catenin (CTNNB1) genes mutations. *J Cancer Res Clin Oncol* 2007;**133:**361-71.

76. Pijnenborg JM, Kisters N, van Engeland M, Dunselman GA, de Haan J, et al. APC, beta-catenin, and E-cadherin and the development of recurrent endometrial carcinoma. *Int J Gynecol Cancer* 2004;**14:**947-56.

77. Moreno-Bueno G, Hardisson D, Sanchez C, Sarrio D, Cassia R, et al. Abnormalities of the APC/beta-catenin pathway in endometrial cancer. *Oncogene* 2002;**21:**7981-90.

78. Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A* 1988;**85:**5166-70.

79. Arango NA, Kobayashi A, Wang Y, Jamin SP, Lee HH, et al. A mesenchymal perspective of Mullerian duct differentiation and regression in Amhr2-lacZ mice. *Mol Reprod Dev* 2008;**75:**1154-62.

80. Jamin SP, Arango NA, Mishina Y, Hanks MC, Behringer RR. Requirement of Bmpr1a for Mullerian duct regression during male sexual development. *Nat Genet* 2002;**32:**408-10.

81. Deutscher E, Hung-Chang Yao H. Essential roles of mesenchyme-derived beta-catenin in mouse Mullerian duct morphogenesis. *Dev Biol* 2007;**307:**227-36.

82. Arango NA, Szotek PP, Manganaro TF, Oliva E, Donahoe PK, et al. Conditional deletion of beta-catenin in the mesenchyme of the developing mouse uterus results in a switch to adipogenesis in the myometrium. *Dev Biol* 2005;**288:**276-83.

83. Tanwar PS, Zhang L, Roberts DJ, Teixeira JM. Stromal deletion of the APC tumor suppressor in mice triggers development of endometrial cancer. *Cancer Res* 2011;**71:**1584-96.

84. Jeong JW, Lee HS, Franco HL, Broaddus RR, Taketo MM, et al. beta-catenin mediates glandular formation and dysregulation of beta-catenin induces hyperplasia formation in the murine uterus. *Oncogene* 2009;**28:**31-40.

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# Chapter 3

## Progesterone inhibits epithelial-to-mesenchymal transition in Endometrial Cancer

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## Abstract:

### Background:

Every year approximately 74,000 women die of endometrial cancer, mainly due to recurrent or metastatic disease. The presence of tumor infiltrating lymphocytes (TILs) as well as progesterone receptor (PR) positivity has been correlated with improved prognosis. This study describes two mechanisms by which progesterone inhibits metastatic spread of endometrial cancer: by stimulating T-cell infiltration and by inhibiting epithelial-to-mesenchymal cell transition (EMT).

### Methodology and principle findings:

Paraffin sections from patients with (n=9) or without (n=9) progressive endometrial cancer (recurrent or metastatic disease) were assessed for the presence of CD4+ (helper), CD8+ (cytotoxic) and Foxp3+ (regulatory) T-lymphocytes and PR expression. Progressive disease was observed to be associated with significant loss of TILs and loss of PR expression. Frozen tumor samples, used for genome-wide expression analysis, showed significant regulation of pathways involved in immunesurveillance, EMT and metastasis. For a number of genes, such as CXCL14, DKK1, DKK4, PEG10 and WIF1, quantitive RT-PCR was performed to verify up- or downregulation in progressive disease. To corroborate the role of progesterone in regulating invasion, Ishikawa(IK) endometrial cancer cell lines stably transfected with PRA (IKPRA), PRB(IKPRB) and PRA+PRB (IKPRAB) were cultured in presence/absence of progesterone (MPA) and used for genome-wide expression analysis, Boyden- and wound healing migration assays, and IHC for known EMT markers. IKPRB and IKPRAB cell lines showed MPA induced inhibition of migration and loss of the mesenchymal marker vimentin at the invasive front of the wound healing assay. Furthermore, pathway analysis of significantly MPA regulated genes showed significant down regulation of important pathways involved in EMT, immunesuppression and metastasis: such as IL6-, TGF-β and Wnt/β-catenin signaling.

### Conclusion:

Intact progesterone signaling in non-progressive endometrial cancer seems to be an important factor stimulating immunosurveilance and inhibiting transition from an epithelial to a more mesenchymal, more invasive phenotype.

## Introduction:

Each year, worldwide, more than 287,000 women develop endometrial cancer making it the most common gynecological cancer in the world and the fourth most common female malignancy in developed countries[1]. Usually endometrial cancer is detected in an early stage and surgery is the cornerstone of treatment. Where there is recurrent or metastatic disease, however, the situation is different. (Neo-)Adjuvant radiation and/or systemic therapy in combination with surgery is usually indicated and in general, progressive disease has a poor prognosis accounting for 74,000 deaths worldwide each year[2, 3]. Prognostic factors for recurrent and metastatic endometrial cancer include surgical FIGO stage, grade of differentiation, histopathological subtype and myometrial and lymphovascular invasion[2, 4-7].

In several types of cancer, the presence of tumor infiltrating lymphocytes (TILs) has been correlated with improved prognosis, and much research has been performed on this topic[8-15]. The rationale is that well differentiated cancer evokes an inflammatory response similar to an acute injury which, after sequential infiltration of different dendritic cell populations, eventually results in T-lymphocyte infiltration[16]. Infiltration of TILs as a positive prognostic factor was first described in cutaneous melanoma, where the presence of TILs was predictive for improved survival[8]. Galon et al. in 2006, showed that infiltration of lymphocytes of the adaptive immune system into the center and invasive margin of colorectal cancer was positively correlated with reduced recurrence and improved survival[10]. In 2009 Kilic et al., showed that high levels of TILs within non-small-cell lung cancer correlated with reduced recurrence and enhanced survival[12]. In ovarian cancer, the presence of intratumoral T-lymphocytes was also positively correlated with improved survival and delayed recurrence of the disease[15]. Furthermore, TILs in ovarian cancer were also associated with increased levels of INF-γ, IL2 and chemokines which indicates T-cell activation and attraction[15].

The presence of TILs has not been extensively investigated in endometrial cancer. In endometrial cancer, infiltration of cytotoxic (CD8+) T-lymphocytes in the area of the lesion has been described as an independent prognostic factor and is positively correlated to disease free- and overall survival[17, 18]. In addition, a high cytotoxic T-lymphocyte/regulatory T-lymphocyte (CD8/FOXP3) ratio has been described to be correlated to improved survival in type I endometrial cancer[17].

Next to the influx of T-lymphocytes into the tumor area, the presence of progesterone receptors (PR) is also described as an important asset in prognosis and treatment of endometrial cancer[19-21]. In well differentiated endometrial cancer PR expression is usually maintained and treatment with medroxyprogesterone acetate (MPA), of those patients with well differentiated disease who chose to preserve fertility, is usually successful[22, 23]. Loss of PR, however, is a negative prognostic factor and is associated with progressive disease in which MPA treatment is usually only temporally successful in 15-20% of cases[24].

Recently, our group has studied the mechanism through which progesterone can induce differentiation during the normal menstrual cycle and can inhibit well differentiated endometrial cancer growth. It was observed that progesterone treatment results in induction of expression of two important inhibitors of Wnt/β-catenin signaling: DKK1 and FOXO1[25, 26]. In endometrial cancer,

activation of Wnt/β-catenin signaling is observed in 30-40% of well differentiated endometrioid carcinomas[27] and progesterone induced inhibition of the Wnt signaling pathway is hypothesized to be an important mechanism to reduce cancer progression[25].

In this study we aimed to investigate the role of progesterone as a direct inhibitor of the migratory capacities of endometrial cancer cells and its role in T-lymphocyte associated inhibition of progressive disease.

## Materials and methods:

### Patient materials:

Primary endometrial carcinoma tissue from women with (n=9) and without (n=9) a known episode of recurrence or metastasis, was obtained from patients treated between 1997 and 2006 in the University Hospital Gasthuisberg, Catholic University Leuven, Belgium. From this point on, non-recurrent disease is referred as non-progressive disease and recurrent/metastatic disease as progressive disease. Histopathological grading, staging and typing were determined according to the guidelines of the WHO and FIGO[28, 29] and all tumors were revised by a pathologist experienced in gynaecopathology (PCE). Patients with an endometrioid type and a FIGO stage I endometrial carcinoma were included. Patients treated with radio- or chemotherapy prior to surgery, using hormonal steroids or with a second malignancy were excluded. Complete clinical history was obtained from all patients and follow-up was revised to date. Specimens were snap-frozen in liquid nitrogen for RNA-isolation or fixed in formalin and embedded in paraffin for immunohistochemistry (IHC). For microarray analysis, from 4 non-progressive and 4 progressive patients, snap frozen tumor specimens were used. These were chosen because they contained > 80% tumor tissue and good quality RNA could be isolated from them. For RT-PCR, 6 non-progressive and 6 progressive snap frozen patient tissue samples were used. For IHC 9 non-progressive and 9 progressive paraffin embedded patient tissue samples were available. Tissue and clinical data collection for the current research study was approved by the Medical Ethical Committee of the University Hospital Gasthuisberg and patients gave written informed consent for tissue collection and clinical data collection for all research purposes.

### Cell culture:

For all cell line experiments, Ishikawa endometrial cancer cell lines stably transfected with PRA (IKPRA-1), PRB (IKPRB-1) or PRA and PRB (IKPRAB-36) (previously described by Smit-Koopman et al.[30]) were cultured and maintained in regular culture medium (DMEM/F12 Glutamax, Invitrogen, Carlsbad, CA, USA) in the presence of 5% Fetal Calf Serum (Invitrogen) supplemented with penicillin and streptomycin (Invitrogen). Neomycin (ICN Biomedicals, Costa Mesa, CA, USA) and hygromycin (Invitrogen) 1:200 were used to maintain selection. For all assays, cells were cultured in DMEM/F12 Glutamax culture medium supplemented with penicillin and streptomycin (Invitrogen), containing 5% charcoal stripped FCS (Invitrogen) with addition of hygromycin and neomycin.

**Immunohistochemistry:**

IHC studies for CD4 (Sanbio BV, Uden, The Netherlands), CD8 (Dako, Glostrup, Denmark), FOXP3 (Natutech, Frankfurt am Main, Germany) and PRA+PRB (Progesterone Receptor Ab-8, Neomarkers, Fremont, CA, USA) were performed on 4 µm paraffin sections on Starfrost-slides (Knittel, Braunschweig, Germany). Prior to incubation with the primary antibody, the slides were deparaffinized in xylene and rehydrated to 70% ethanol. For CD4+ and CD8+ T-lymphocyte staining, slides were microwaved at 850 Watt in Tris/EDTA pH 9.0 for 15 min. Endogenous peroxidase activity was blocked with 30% $H_2O_2$ in PBS for 5 min. Primary antibodies were applied at respectively 1:160 (CD4) and 1:200 (CD8) in Tris/HCl pH8.0 and incubated at room temperature for 30 min. After washing with Tris/HCl pH8.0, sections were incubated for 30 min. at room temperature with biotinylated secondary antibody (Dako, 1:400). After washing with Tris/HCL, the substrate Diaminobenzidine (Dako) was used for visualization of antigen–antibody reactivity.

For FOXP3, slides were blocked (peroxidase deactivation) for 20 min at room temperature (RT) in 30% $H_2O_2$ in methanol and boiled (antigen retrieval) in a citrate-buffer pH6.0 for 15 min. Primary antibody was applied at 1:25 and incubated at 4°C overnight. After washing with PBS, slides were incubated for 30 min. with a secondary rabbit-anti-rat antibody (DAKO, 1:150) and incubated for 30 min. with AB-complex (Dako). The substrate Diaminobenzidine (Dako) was used for visualization of antigen–antibody reactivity.

For PRA+PRB staining, endogenous peroxidase activity was blocked for 5 min at RT in a 10% $H_2O_2$ in methanol solution and the slides were microwaved (antigen retrieval) in a microwave-oven at 850 Watt in 10nM citric acid buffer pH6.0 (DAKO) for 15 min. After cooling to room temperature slides were washed with PBS and blocked for 30 min with 0.3% BSA/PBS. Primary antibody was applied at 1:50 and incubated at 4°C overnight. After washing with PBS, slides were incubated for 30 minutes with a biotinylated secondary goat-anti-mouse antibody (Dako, 1:400). After the second wash the slides were incubated for 30 min with AB-complex (Dako, 1:1:50). The substrate Diaminobenzidine (Dako) was used for visualization of reactivity. All slides were counterstained with hematoxylin for 30s, then dehydrated and mounted.

For Vimentin staining, a wound-healing assay was performed in 2-well chamber slides (Lab-Tek, Thermo Fisher Scientific, Waltham, MA, USA), in the presence and absence of 1 nM medroxy-progesterone acetate (MPA), and terminated after 48 hr. The cells were washed three times with PBS, fixed with 4% formaldehyde/PBS for 15 minutes and permeabilized with 0,3% Triton100/PBS for 5 minutes. After washing, endogenous peroxidase activity was blocked with 10% $H_2O_2$ in methanol for 5 minutes. Slides were washed and then blocked for 30 minutes with 0.3% BSA/PBS. The anti-vimentin antibody (Invitrogen) was applied at 1:50 and the slides were incubated for 30 minutes at room temperature. After washing with PBS, slides were incubated with a GFP-fluorescent goat-anti-mouse secondary antibody (Invitrogen) at 1:500. After washing, the slides were incubated for 5 minutes with DAPI Nucleic Acid Staining Solution (Invitrogen) for nuclear staining. After termination of the reaction with $dH_2O$, the slides were mounted and fluorescent images were taken with the Axioplan 2 Imaging Fluorescent Microscope (Carl Zeiss AG, Jena, Germany).

### Counting TILs:

After staining, the slides were scanned with the NDP slide scanner (Hamamatsu, Hamamatsu City, Japan) and CD4, CD8 and FOXP3 positive tumor infiltrating lymphocytes (TILs) were counted using Image J software (National Institutes of Health, Bethesda, MD, USA). The number of TILs was determined inside the tumor (Intratumoral), at the tumor edge (Tumor Edge) and at the endometrial/myometrial border (EM). The complete tumor edge and endometrial/myometrial border were evaluated for the presence of TILs. The intratumoral count was performed by counting the TILs in 10 different randomly picked areas (1170µm x 932µm) chosen by an independent investigator, thereby eradicating observer bias.

### WST1 assay:

For the WST1 proliferation assay, IKPRA-1, IKPRB-1 and IKPRAB-36 cell lines were cultured in the absence or presence of MPA in a 96 well plate (Corning Costar, Cambridge, MA, USA). At time 0, the cells were incubated with cell proliferation reagent WST1 (Roche, Basel, Switzerland) for 3 hours at 37 °C and absorbance was measured with the Microplate Reader (BIORAD, model 550, Hercules, CA, USA). After baseline measurement the cell lines were cultured in the presence and absence of 1 nM MPA for 96 hours and at 96 hours, the WST1 assay was repeated.

### Migration assays:

For the wound-healing assay, IKPRA-1, IKPRB-1 and IKPRAB-36 cell lines were cultured in a 6-well plate (Corning Costar). After inducing the wound, cells were incubated with 1 nM MPA for 96 hours. Wound healing was verified every 24 hr by photography, and analyzed by measuring closure of the wound.

For the modified Boydon assay, cells were seeded in the upper well of a modified Boydon chamber (Transwell, 8 µm pores, 24 mm inserts, 6 well plate, Corning Costar) at 2.5 x $10^5$ cells per well in the presence or absence of 1nM MPA. Furthermore as a control, cells were cultured in a Boyden chamber in the presence or absence of 1nM MPA in combination with 100 nM of the anti-progestagin Org31489 (Organon, Oss, The Netherlands). After 96 hours, cells that had migrated through the filter into the lower well or to the bottom of the insert were trypsinized and counted under the microscope.

### Western blotting:

IKPRA-1, IKPRB-1, IKPRAB-36 and IKLV-8 cell lines were cultured in the absence or presence of 1 nM MPA for 96 hrs and subsequently lysed at 0°C in Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) for 5 minutes. Then the cells were scraped, centrifuged at 14.000 rpm for 10 minutes and the supernatant was removed. The protein concentration was calculated using the Protein Assay Kit (Pierce, Thermo Scientific, Rockford, IL, USA) and of each sample 4.5 µg protein in 30 µL lysisbuffer + BSA was loaded on a 10% SDS-PAGE gel. Western blotting was performed according to standard procedures. The blotting paper was blocked for 30 minutes at RT with Blocking Buffer (LI-COR

Biotechnology, Lincoln, NE, USA) and then incubated overnight at 4°C using rabbit polyclonal anti-hFOXO1 antibody (1:5000, Bethyl Laboratories, Montgomery, TX, USA) in Blocking Buffer (LI-COR Biotechnology). Next, the blotting membrane was incubated with the secondary goat-anti-rabbit IgG (IRDye 800CW, 1:5000, LI-COR Biotechnology) for 30 minutes at RT and washed. As a loading control, the membrane was incubated for 30 minutes with the monoclonal anti-β-actin (1:1000, Sigma-Aldrich, Saint Louis, MO, USA), washed with PBS and incubated for 30 minutes with the secondary goat-anti-mouse IgG (IRDye 680CW, 1:5000, LI-COR Biotechnology). The specific protein bands were detected using the Odyssey Scanning System (LI-COR Biotechnology).

**RNA-isolation, gene expression analyses and quantitative real-time RT-PCR:**

Patient tissue samples were sectioned (5 µm, cryostat) and every 10$^{th}$ section was HE stained and revised by the pathologist (PCE) to assess tumor load. Only sections containing >80% tumor were lysed in Trizol (Invitrogen) and sonified for 1 min. The PRA and PRB expressing Ishikawa cell line (IKPRAB-36) was cultured for 48h in the absence or presence of 1nM MPA (n = 3), placed on ice and lysed in Trizol (Invitrogen).

Phase separation was accomplished with 0.2 ml chloroform and centrifugation for 15 min. The supernatant was transferred and isopropanol was added for RNA precipitation. The precipitated RNA was washed with 75% ethanol. All RNA was cleaned with the Rneasy Minelute cleanup kit (Qiagen, Venlo, The Netherlands). Amount and quality of the RNA was assessed by using the Nanodrop (Nanodrop, Wilmington, DE, USA) and Bio-analyzer (Aligent, Santa Clara, CA, USA).

RNA isolated from patient and cell line material was labeled according to Affymetrix labeling protocols and labeled RNA was applied to genome-wide expression arrays (Affymetrix U133plus2 GeneChips containing 54,614 probe sets, representing approximately 47.000 transcripts (Affymetrix, Santa Clara, CA, USA)). Using RMA (Robust Multi-array Analysis[31]), normalization of raw data was performed to be able to produce gene lists and eventually calculate significantly regulated genes using SAM (Stanford University, Stanford, CA, USA[32]). Lists of SAM regulated genes (1.25 fold or more; delta-values resembling $p<0.05$) were loaded in the Ingenuity pathway assist software to assess the involvement of different biological pathways (Ingenuity, Redwood City, CA, USA). For the patient materials raw lists of regulated genes (1.25 fold or more) were loaded in Ingenuity.

All micro-array data is MIAME compliant and raw data has been deposited in the MIAME compliant GEO database under series: GSE29437 (consisting of GSE29435: cell line data; and GSE29436: patient data).

Genes for quantitative real-time RT-PCR were identified by micro-array analysis and pathway analysis. RNA was transcribed into cDNA with the use of the Affymetrix one-cycle cDNA synthesis kit (Affymetrix). For identified genes, primers were ordered and tested (a list of primers is included in Table S1). The housekeeping gene β-actin was used as a reference gene. RT-PCR was performed and analyzed using the CFX RT-PCR system (Bio-Rad, Veenendaal, The Netherlands).

### Statistics:

For the statistical analyses of the CD4+, CD8+ and FOXP3+ cell counts, modified Boyden chamber assay data, WST1 assay data and RT-PCR data, SPSS 15.0 was used (IBM, Armonk, NY, USA). For normal distributed data a t-test and for skewed data a Mann-Whitney U-test was performed to assess P-values. A P-value < 0.05 was considered statistically significant. To calculate the p-value of regulated pathways, Ingenuity pathway assist software uses a Fisher's exact test.

## Results:

**Table 1: Clinical characteristics of the included patients.**

| | | | *Non-progressive (n=9) Patients 1-9* | *Progressive (n=9) Patients 10-18* | *P-value* |
|---|---|---|---|---|---|
| **Age - year** | | Mean | 68,5 | 68,6 | p = 0,606 |
| | | Range | 54-85 | 59-73 | |
| **BMI** | | Mean | 28,3 | 32 | p = 0,284 |
| | | Sd | 6,1 | 4,7 | |
| **Histological type** | no. (%) | Endometrioid<br>Mixed | 9 (100)<br>0 ( -- ) | 8 (88,9)<br>1 (11,1) | |
| **FIGO stage** | no. (%) | Ia | 4 (44,4) | 7 (77,8) | |
| | | Ib | 5 (55,6) | 2 (22,2) | |
| **Tumor grade** | no. (%) | 1 | 2 (22,2) | 5 (55,6) | |
| | | 2 | 3 (33,3) | 1 (11,1) | |
| | | 3 | 4 (44,5) | 3 (33,3) | |
| **Current status** | no. (%) | NED | 8 (88,9) | 3 (33,3) | |
| | | DOD | 1 (11,1) | 6 (66,7) | |
| **Recurrence** | no. (% | No | 9 (100) | 0 ( -- ) | |
| | | Yes | 0 ( -- ) | 9 (100) | |
| **Metastasis** | no. (%) | No | 9 (100) | 5 (55,6) | |
| | | Yes | 0 ( -- ) | 4 (44,4) | |
| **Chemotherapy** | no. | | 0 | 0 | |
| **Radiotherapy** | no. | | 0 | 1 | |

Table 1 shows the characteristics of the patients included in the study. A p-value of < 0.05 was considered as statistically significant. BMI= body mass index; NED= no evidence of disease; DOD=death of disease.

### Patient characteristics (Table 1):

Patients with (n=9) and without (n=9) progressive endometrial cancer were included. All included patients underwent primary total abdominal- or laparoscopically assisted vaginal hysterectomy and a bilateral salpingo-oophorectomy combined with lymph node removal. None of the women received chemotherapy and only one woman in the progressive disease group was given radiotherapy after surgery. Histopathological subtypes were endometrioid (n=17) and mixed endometrioid/mucinoid (n=1). Tumor grades were 1 (n=7), 2 (n=4) and 3 (n=7) and FIGO stages were Ia (n=11) and Ib (n=7). In the progressive disease group all 9 patients had one or more episodes of local recurrence and 4 patients developed one or multiple distant metastases. Recurrences were vaginal, pelvic or (retro)peritoneal, and metastatic sites were the lungs (n=3), liver (n=1), spleen (n=1) and brain (n=1). Clinical follow-up to date was available for all patients. In the non-progressive group 8 patients are currently free of disease and 1 patient died in follow-up. In the progressive disease group 3 patients are free of disease and 6 patients died from their endometrial cancer related disease. Patient characteristics are detailed in Table 1.

### Progesterone receptor status and detection of CD4+ T-helper, CD8+ cytotoxic T-cells and FOXP3+ regulatory T-cells in non- progressive and progressive disease

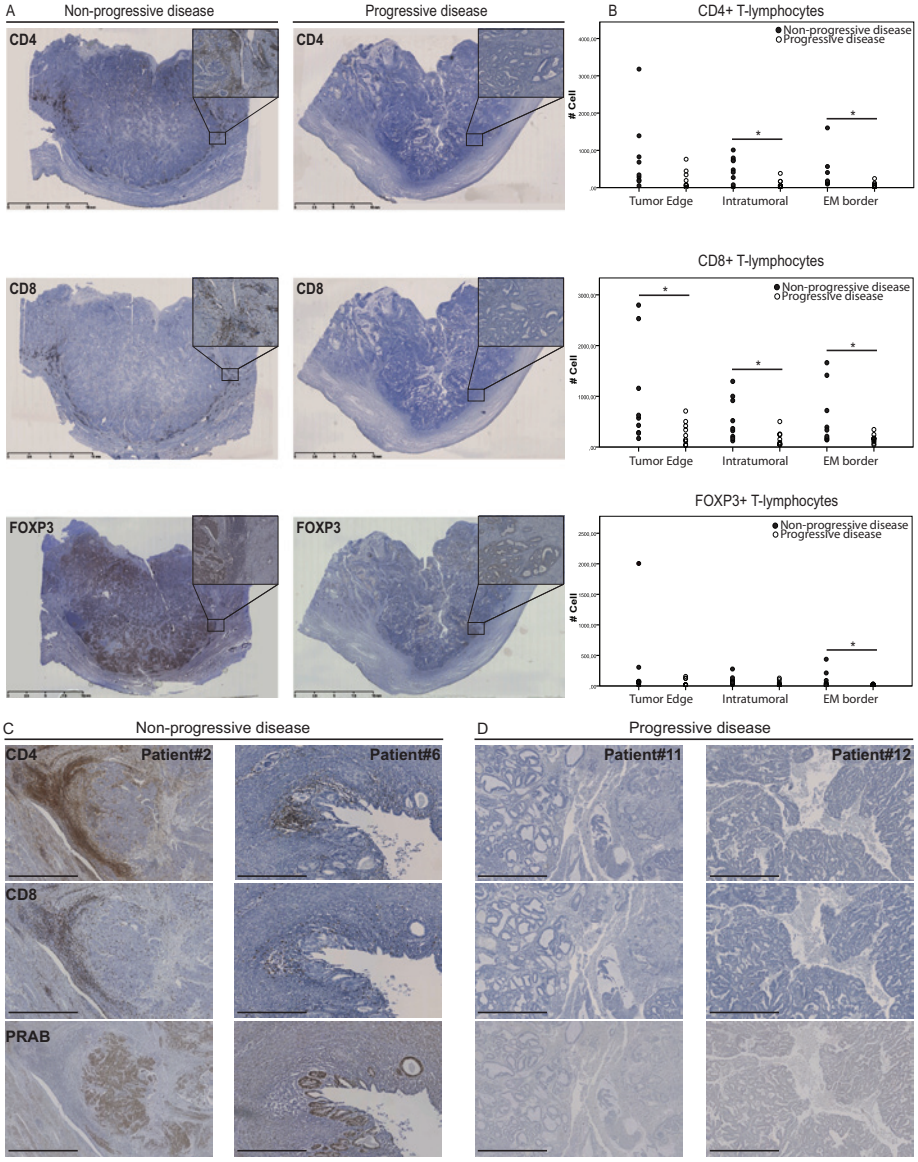
The presence of tumor infiltrating lymphocytes has been correlated to prolonged survival in endometrial cancer[17, 18]. Furthermore, loss of progesterone receptor (PR) expression in endometrial cancer has been found to be a risk factor for progressive disease[33]. In order to substantiate the relationship between intact PR signaling and the presence of infiltrating lymphocytes in non-progressive disease, immunohistochemical staining and, when appropriate, quantitative measurements were performed.

As exemplified in Fig. 1A, in progressive disease immunohistochemical staining for CD4+, CD8+ and FOXP3+ T-lymphocytes seems reduced as compared to staining in non-progressive disease. Quantification of the number of CD4+, CD8+ and FOXP3+ T-lymphocytes in progressive disease indeed confirmed a lower number of positive cells located on the endometrial-myometrial border (Fig 1B, EM), at the edge of the tumor (Fig 1B, Tumor Edge) and within the tumor (Fig. 1B, Intratumoral). Whether the reduced cell counts were significantly different between the non-progressive and progressive endometrial cancer tissues is indicated in the Figure (Fig. 1B).
Furthermore, reviewing consecutive sections in non-progressive disease for expression of progesterone receptors (PR) revealed that the presence of CD4+ and CD8+ T-lymphocytes was positively correlated with the presence of PR staining (Fig. 1C and 1D).

### Genome-wide expression analyses of primary endometrial carcinoma tissue

To investigate whether the correlation between PR signaling and the presence of tumor infiltrating lymphocytes could indicate a causative relationship, a genome-wide mRNA expression analysis on snap-frozen primary endometrial carcinoma specimens from 4 patients without and 4 patients with progressive disease was performed.

**Figure 1: Expression and histological distribution of PRA+PRB and CD4+, CD8+ and Foxp3+ T-lymphocytes in primary endometrial carcinoma specimens.**




A: Overview of immunohistochemical staining for CD4, CD8 and FOXP3 in primary endometrial cancer specimens in non-progressive disease (n=9) compared to progressive disease (n=9) (magnification 0,4x, inlay 10x). Non-progressive disease shows pronounced staining, whereas progressive disease shows reduced staining. The scale-bar represents 10 mm. B: Quantification of CD4, CD8 and FOXP3 cell counts on the tumor edge (Tumor Edge), in the tumor (Intratumoral) and on the endometrial-myometrial border (EM border). *indicates a p-value <0.05 (Mann-Whitney U-test). C and D: Representative non-progressive (C) and progressive (D) patient tissues were stained for CD4, CD8 and PRA+PRB and show a positive correlation between the presence of TILs and the expression of PR. Magnification is 5x and the scale-bar represents 1 mm. Patients 6 and 11 were both included in the micro-array analyses. Furthermore patient 11 had only recurrent disease, while patient 12 had recurrent and metastatic disease.

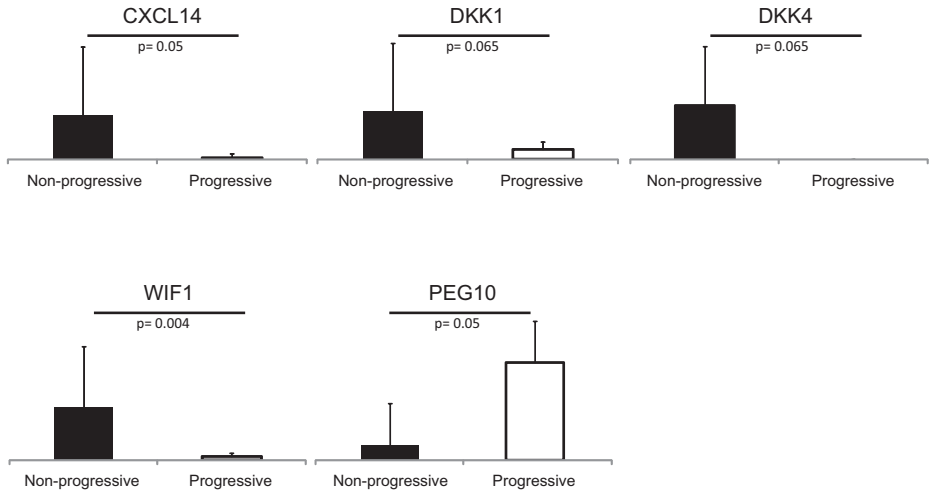
At the individual gene level it was observed that a marked number of chemokines and cytokines were differentially regulated between non-progressive and progressive disease (Table S2). For example, the chemokines CCL21 (-1.5x), CXCL9 (-2.9x), CXCL10 (-2.1x) and CXCL14 (three data sets present: -33.0x; -20.5x; -6.4x, respectively) were all down regulated in progressive disease while the cytokines IL8 (2.0x; 5.7x; 9.5x) and IL32 (1.9x) were up-regulated in progressive disease (Table S2). Furthermore, earlier work from our group has indicated activation of Wnt/β catenin signaling in progressive disease[25] and in agreement with this a number of Wnt/β-catenin inhibitory- and target genes were lost from progressive disease (DKK1, DKK4 and WIF1) (Table S2).

Interestingly, a number of the above mentioned genes which were down-regulated in progressive disease, have been described in literature to be up-regulated by progesterone (CXCL14[34] , DKK1[25], MMP7[35] and SFRP4[36]). This is in agreement with the finding that PR expression (at protein and mRNA expression level (Fig. 1C and 1D and Table S2) is down regulated in progressive disease.

Upon reviewing pathways regulated between non-progressive and progressive disease, regulation of a number of pathways involved in carcinogenesis and invasive disease and involved in immunosurveillance was found to be significantly regulated: Integrin Signaling, Molecular Mechanisms of Cancer, Antigen Presentation Pathway, Non-Small Cell Lung Cancer Signaling, IGF-1 Signaling, Role of Tissue Factor in Cancer, Leukocyte Extravasation Signaling, ERK/MAPK Signaling, Colorectal Cancer Metastasis Signaling (which includes Wnt/β catenin signaling), FGF Signaling, FAK Signaling, etc (the complete list of regulated pathways and their consecutive p-values can be accessed from Table S3).

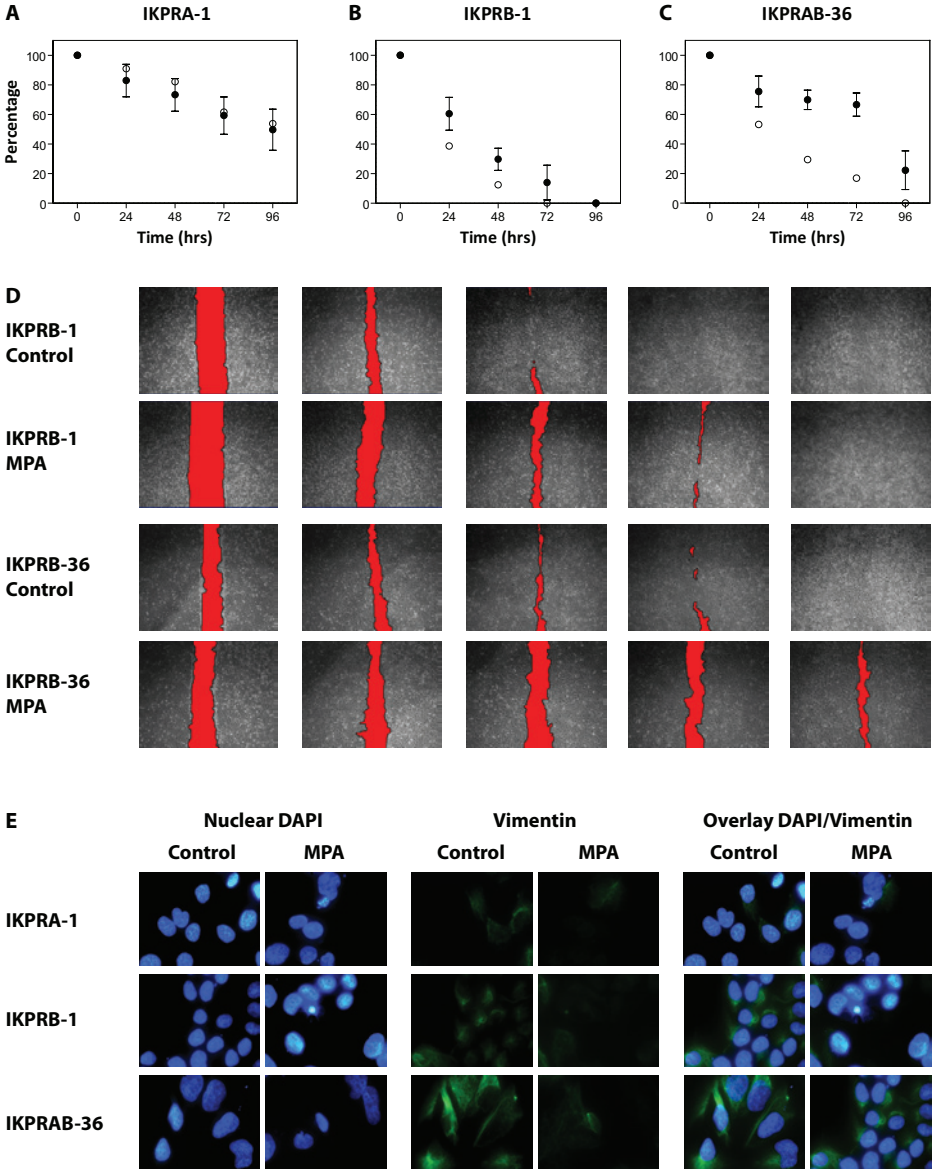
For a number of genes (CXCL14, DKK1, DKK4, PEG10 and WIF1) a quantitative real-time RT-PCR was performed in order to verify regulation (Fig. 2).

**Figure 2: RT-PCR results of genes of interest in the patient samples.**




CXCL14, DKK1, DKK4, WIF1 and PEG10 were selected from the micro-array results and verified with real time RT-PCR. Significance was calculated using a Mann-Whitney U-test. A p-value of 0.05 was considered to be statistically significant.

**Figure 3: Progesterone induced inhibition of migration in a wound-healing assay**.




IKPRA-1 (A), IKPRB-1 (B) and IKPRAB-36 (C) cells were cultured in the absence (white bullets) or presence (black bullets) of 1 nM MPA and used for a wound-healing assay (n = 3) and closure of the wound was measured as a percentage of total closure (100% means the wound is open, 0% means the wound has closed). D shows representative images of the process of wound-healing with in red the wound. E shows IF for nuclei (DAPI) and vimentin expression on the invasive front of the manually inflicted wound. In this figure, the wound was always situated on the right side.

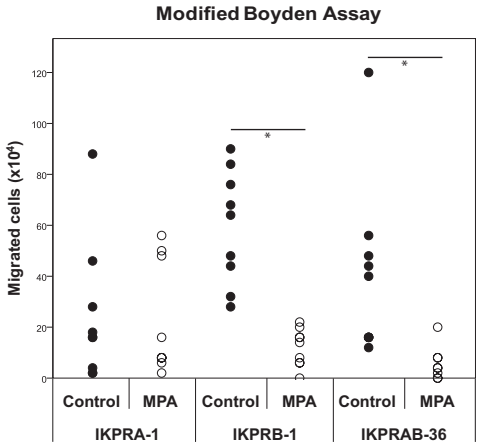
## Effect of progesterone on migration of the Ishikawa endometrial cancer cell lines

In order to further corroborate the possible role for progesterone in regulating invasion, Ishikawa endometrial carcinoma cell lines stably transfected with PRA, PRB, or PRA and PRB[30] were cultured in the presence or absence of MPA for varying periods of time and used in two different experiments measuring cell migration. To verify cell proliferation during the different experiments a WST1 proliferation test was performed which showed that within the indicated timeframe no significant differences in proliferation could be detected between cells incubated with or without MPA.

In Figure 3, different Ishikawa cell lines were subjected to a wound-healing assay in the presence or absence of MPA (1nM) for up to 96h. It was observed that, in the stably PRB expressing (IKPRB-1) and PRA+PRB expressing (IKPRAB-36) Ishikawa cell lines, MPA inhibited closure of the manually inflicted wound (Fig. 3A-D). Furthermore, when we stained the edge of the wound for the mesenchymal marker vimentin, it was observed that in the presence of MPA vimentin expression was clearly reduced (Fig. 3E). Next to this detail on expression of vimentin, the overall vimentin levels were decreased in IKPRB-1 and IKPRAB-36 cell lines incubated with 1 nM MPA. It was also observed that in the stably PRA expressing (IKPRA-1) Ishikawa cell line, neither wound healing nor vimentin expression was affected by MPA (Fig. 3A and 3E).

In Figure 4, another approach was used to study the migratory capacity of different Ishikawa cell lines in the presence or absence of progesterone. It was observed that for IKPRB-1 as well as IKPRAB-36 cells, migration in a modified Boyden chamber was inhibited in the presence of progesterone. Furthermore, for the IKPRA-1 cell line such a differential regulation of migration under the influence of MPA was not observed.

**Figure 4: Invasion of PR positive Ishikawa EC cell lines.**




IKPRA-1, IKPRB-1 and IKPRAB-36 cells were cultured in the absence (black dots) or presence (white dots) of 1 nM MPA in a modified Boyden chamber. After 96 hours, cells that had migrated through the pores of the upper well were counted. The figure represents three independent experiments performed in triplicate. *indicates a p-value of <0.05 (Mann-Whitney U-test).

### Genome-wide expression analysis of Ishikawa endometrial cancer cell line

To further document progesterone-induced inhibition of cellular migration and to investigate the involvement of progesterone signaling in T-lymphocyte infiltration, IKPRAB-36 cells were cultured for 48h in the presence or absence of 1nM MPA and used for genome-wide expression analysis. It was observed that 1616 genes were significantly regulated by progesterone in the IKPRAB-36 cell line (1029 up-regulated, 587 down-regulated, Table S4).

Using Ingenuity pathway analysis of significantly regulated genes, the following pathways were observed to be regulated by progesterone (the complete list of regulated pathways and their consecutive p-values can be accessed from Table S5): IGF-1 signaling, Neuregulin signaling, TNFR1 signaling, P13K signaling in B-lymphocytes, VDR/RXR signaling, Acute Phase Response signaling, Hepatic Fibrosis / Hepatic Stellate Cell activation, Molecular Mechanisms of Cancer (which includes Wnt/β-catenin and TGF-β signaling), TGF-β signaling, Axonal Guidance Signaling etc. Interestingly, it was noted that 41/67 pathways observed to be significantly regulated by progesterone in the cell line were also found to be significantly regulated between non-progressive and progressive disease (see Table S6). Furthermore, it was also noted that a number of pathways specifically involved in transition from a epithelial state to a mesenchymal state (EMT) was significantly regulated by progesterone and in the endometrial cancer samples: EGF signaling ($p=0.029$), IGF-1 signaling ($p=0.0000006$), IL-6 signaling (0.013), ILK signaling ($p=0.018$), PDGF signaling ($p=0.03$), TGF-β ($p=0.003$), VEGF signaling ($p=0.022$) and Wnt/β-catenin signaling ($p=0.036$). In Figure 5A and B, MPA-induced gene regulation in Wnt/β-catenin and TGF-β signaling is shown. Next to this, a heat map confirmed a major overlap between gene regulation by MPA and differential gene expression between non-progressive and progressive disease (Table S7).

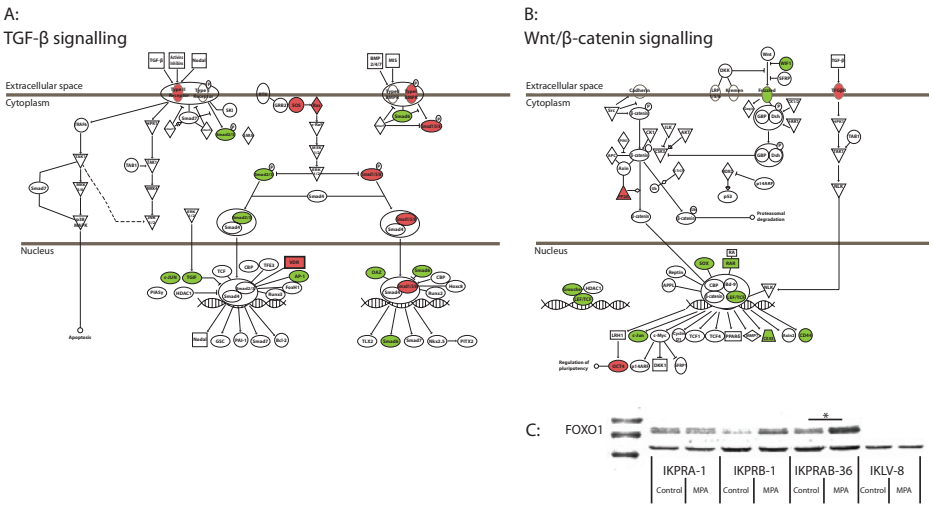
Regulation of the Wnt signaling pathway was further confirmed by showing progesterone induction of the Wnt inhibitor FOXO1 at the protein level (Fig. 5C).

## Discussion:

In general, patients with endometrial cancer have a good prognosis since early diagnosis is frequent and the disease has usually not spread beyond the uterus. However, the prognosis for recurrent or metastatic endometrial cancer remains poor and in order to improve therapy it is vital to understand the processes which inhibit and stimulate cancer progression.

Infiltration of T-lymphocytes into the region of the lesion, for example, is an anticancer signal which helps to confine a tumor until cancer-induced T-cell death establishes tumor immune tolerance opening the road to progression. The transition of an epithelial phenotype towards a more mesenchymal phenotype is a subsequent step which leads to further progression to invasive disease. Central to this epithelial to mesenchymal transition (EMT) is the activation of important signaling pathways such as Wnt/β-catenin and TGF-β[37]. Activation of these pathways results in induction of Snail1/2 induced transcription, eventually causing degradation of the basement membrane by induction of matrix metalloproteinases, loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as vimentin[37].

**Figure 5: MPA induced regulation of TGF-β and Wnt/β-catenin signaling in the IKPRAB-36 cell line.**




A and B: In these pathways a green color represents down regulation by MPA and a red color represents up regulation by MPA. Signaling pathways were provided by Ingenuity Pathway Assist Software© and individual gene expression levels are available in Table S4. C: Western blot showing FOXO1 expression in the IKPRA-1, IKPRB-1, IKPRAB-36 and IKLV-8 cell lines cultured in the absence (control) or presence (MPA) of 1 nM MPA. *indicates significant regulation in the micro-array analysis (Table S4).

In the current investigations non-progressive and progressive primary endometrial cancer tissues were compared and it was observed that progression of disease was characterized by 1. Loss of progesterone signaling, 2. Loss of CD4, CD8 and FOXP3 T-lymphocytes driven immunosuppression and 3. Modulation of genes and pathways reminiscent of EMT. The aim of the present investigations was to assess the role of decreased progesterone signaling in progressive disease, and more particularly in relation to loss of immunosuppression and transition from an epithelial phenotype to a more invasive mesenchymal phenotype.

## Loss of PR expression correlates with loss of immunosupression and increased EMT in progressive disease

Measuring tumor infiltrating lymphocytes (TILs) in primary endometrial cancer tissues from non-progressive and progressive disease indicated that in patients with non-progressive endometrial cancer, TILs were abundantly present. This is in agreement with studies by Kondratiev et al. in 2004 [18] and De Jong et al. in 2009[17], which showed that high levels of CD8+ T-lymphocytes were associated with improved disease free survival. Furthermore, the presence of several chemokines (CCL21, CXCL9, CXCL10, CXCL14, IL8 and IL32) indicated that there is an active process which directs TILs to the site of the lesion[38]. Interestingly, a number of these chemokines are up-regulated during the secretory phase of the menstrual cycle when progesterone levels are increased (CCL21: 1.5-fold up, CXCL10: 1.3-fold up and CXCL14: 90-fold up;[39]). Furthermore, CXCL14 has also been described by other groups to be a progesterone induced gene in the endometrium involved in

chemo-attraction of uterine natural killer cells to the epithelial glands[34]. In summary, this indicates a putative role for progesterone signaling in attracting TILs in non-progressive endometrial cancer. In the patient tissues which were used in the current investigations, progesterone receptor expression was lost from progressive disease. The fact that hormonal control of a tissue is lost upon progressive malignant transformation is not a new finding and besides loss of PR expression in endometrial cancer[20] this has also been described for other cancer types like breast cancer (loss of estrogen signaling[40]) and prostate cancer (loss of androgen signaling[41]) as well.

According to previous work from our group, besides stimulating TILs, progesterone can inhibit Wnt/β-catenin signaling and loss of progesterone signaling may be involved in tumor onset and progression towards a more invasive disease[21, 25, 42, 43]. Interestingly, upon reviewing gene expression profiles obtained from progressive and non-progressive endometrial cancer, a number of inhibitors of Wnt/β-catenin signaling were indeed found to be down-regulated in progressive disease (DKK1, DKK4 and WIF1). These findings are in accordance with the hypothesis that Wnt/β-catenin signaling becomes activated through loss of PR signaling, thus accommodating progressive disease[25]. Down-regulation of the Wnt/β-catenin signaling inhibitor WIF1, in this respect, is of interest because down regulation of WIF-1 in prostate cancer cells was observed to be associated with an increased capacity for cell migration and invasion[44]. In keeping with this, in colorectal cancer, overexpression of activated nuclear β-catenin (the hallmark of activated Wnt/β-catenin signaling) is located at the invasive front of the tumor[45] and in colorectal cancer cell lines, activation of β-catenin directly induces EMT[46].

PEG10 was found to be significantly up regulated in progressive disease. Interestingly, PEG10 is a biomarker for progressive development and invasion of hepatocellular carcinoma, gallbladder adenocarcinoma and acute lymphoid leukemia and is found to be regulated by androgens[47-50]. Next to this, PEG10 and IL10 expression is activated by ligation of CCL10-CCR7 and CXCL13-CXCR5 in B-cell acute lymphatic leukemia, and PEG10 contributes to the up-regulation of IL10, which can lead to impairment of the cytotoxicity of CD8+ T-lymphocytes[51]. It was observed that CXCL13 (3,17x) and PEG10 (9,38x and 4,38x, p=0,05) were both up-regulated in progressive disease and possibly this up-regulation can contribute to impairment of the T-lymphocyte mediated anti-tumor response in progressive disease.

Upon reviewing other pathways which were differentially expressed between non-progressive and progressive endometrial cancer, significant up-regulation of a number of pathways involved in progression towards a more mesenchymal phenotype was noted (Table S3). IL8 signaling is one of those regulated pathways and IL8 itself was found to be up regulated 9.5-fold in progressive disease. These data are in line with literature showing that IL8 is a progesterone down-regulated gene[52] and that high levels of IL8 correlate with endometrial metastatic disease[53].

### MPA inhibits EMT in the Ishikawa endometrial cancer cell line.

In order to further substantiate the above finding that loss of progesterone signaling in progressive disease may play a role in diminished T-cell infiltration and induction of EMT, progesterone signaling in the well differentiated Ishikawa endometrial cancer cell line was investigated.

Although both PRA and PRB can activate transcription of target genes in response to progesterone, PRA and PRB have different transcriptional activities[54]. It has been documented that PRB is a stronger activator of transcription than PRA and PRA is thought to be a dominant repressor of PRB[55]. Next to this, the difference in transcriptional activity is further explained by the recruitment of different cofactors by PRA and PRB[56, 57].

In the present study, it was observed that culture of the IKPRB-1 and IKPRAB-36 endometrial cancer cell line, but not IKPRA-1, in the presence of MPA resulted in inhibition of migration and down regulation of the mesenchymal marker vimentin at the edge of a manually inflicted wound.

These findings suggest that progesterone, in vitro, can inhibit cancer cell migration due to inhibition of EMT. Assessment of pathways involved in EMT showed progesterone modulated down regulation of EGF, IGF-1, IL-6, Integrin/ILK, PDGF, TGF-β, VEGF and Wnt/β-catenin signaling. Interestingly, all of these pathways were also observed to be modulated in progressive disease (Table S6). As shown, many of the observed altered signaling pathways in the patient samples (Table S3) were also significantly altered in the Ishikawa cell line, when incubated with or without progesterone (Table S5). In the Ishikawa culture obviously no tumor infiltrating lymphocytes are present and it is only progesterone signaling that contributes to these changes in signaling. Therefore we conclude that regulation of signaling pathways in patient samples can not only be attributed to the presence or absence of tumor infiltrating lymphocytes, but also to changes in progesterone receptor signaling.

Progesterone inhibition of TGF-β signaling and induction of TGF-β signaling in progesterone insensitive progressive disease is an interesting finding because enhanced TGF-β signaling has been shown to be a very potent immunosuppressant signal used in transplantation medicine. Several agents inhibiting TGF-β signaling (anti-TGF-beta antibodies, small molecule inhibitors of TGF-beta, Smad inhibitors) are in the early stages of development aiming to alleviate immunosuppression during carcinogenesis[58]. Furthermore, neutralizing TGF-β resulted in a CD8+ T-lymphocyte anti-tumor immune response in mouse models[59].

Enhanced TGF-β signaling is also of interest because it has been described as an important major driving force of EMT. Reviewing the pathway in more detail revealed for example up regulation of cell adhesion molecule L1CAM. For L1CAM, regulation of transcription by TGF-β signaling has been described[60], but, interestingly, in colorectal cancer L1CAM has also been shown to be a target gene of Wnt/β-catenin signaling and expression of L1CAM was found to co-localize with β-catenin in the invasive front of the tumor[61]. Recently, for endometrial cancer similar observations have been described confirming promoter-binding sites for the Wnt/β-catenin inducing transcription factor LEF-1 and, interestingly, also for the EMT inducing transcription factors SNAI1 and SNAI2[60].

In summary, intact progesterone signaling in non-progressive endometrial cancer seems to be an important factor stimulating immunosuppression and inhibiting transition from an epithelial to a more mesenchymal, more invasive phenotype.

## References


1. Ferlay J, Shin H-R, Bray F, Forman D, Mathers C, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer* 2010;**127:**2893-917.
2. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, et al. Endometrial cancer. *Lancet* 2005;**366:**491-505.
3. Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics, 2010. *CA Cancer J Clin* 2010;**60:**277-300.
4. Cohn DE, Horowitz NS, Mutch DG, Kim S-M, Manolitsas T, et al. Should the Presence of Lymphvascular Space Involvement Be Used to Assign Patients to Adjuvant Therapy Following Hysterectomy for Unstaged Endometrial Cancer? *Gynecologic Oncology* 2002;**87:**243-6.
5. Jolly S, Vargas CE, Kumar T, Weiner SA, Brabbins DS, et al. The impact of age on long-term outcome in patients with endometrial cancer treated with postoperative radiation. *Gynecologic Oncology* 2006;**103:**87-93.
6. Kosary CL. FIGO stage, histology, histologic grade, age and race as prognostic factors in determining survival for cancers of the female gynecological system: an analysis of 1973-87 SEER cases of cancers of the endometrium, cervix, ovary, vulva, and vagina. *Semin Surg Oncol* 1994;**10:**31-46.
7. Lindauer J, Fowler JM, Manolitsas TP, Copeland LJ, Eaton LA, et al. Is there a prognostic difference between depth of myometrial invasion and the tumor-free distance from the uterine serosa in endometrial cancer? *Gynecologic Oncology* 2003;**91:**547-51.
8. Clemente CG, Mihm MC, Jr., Bufalino R, Zurrida S, Collini P, et al. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* 1996;**77:**1303-10.
9. de Vos van Steenwijk PJ, Heusinkveld M, Ramwadhdoebe TH, Löwik MJ, van der Hulst JM, et al. An Unexpectedly Large Polyclonal Repertoire of HPV-Specific T Cells Is Poised for Action in Patients with Cervical Cancer. *Cancer Research* 2010;**70:**2707-17.
10. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;**313:**1960-4.
11. Hiraoka N. Tumor-infiltrating lymphocytes and hepatocellular carcinoma: molecular biology. *International Journal of Clinical Oncology* 2010**:**1-8.
12. Kilic A, Landreneau RJ, Luketich JD, Pennathur A, Schuchert MJ. Density of Tumor-Infiltrating Lymphocytes Correlates with Disease Recurrence and Survival in Patients with Large Non-Small-Cell Lung Cancer Tumors. *J Surg Res* 2009.
13. Le DT, Ladle BH, Lee T, Weiss V, Yao X, et al. CD8+Foxp3+ tumor infiltrating lymphocytes accumulate in the context of an effective anti-tumor response. *International Journal of Cancer* 2010**:**n/a-n/a.
14. Leffers N, Fehrmann RSN, Gooden MJM, Schulze URJ, ten Hoor KA, et al. Identification of genes and pathways associated with cytotoxic T lymphocyte infiltration of serous ovarian cancer. *Br J Cancer* 2010;**103:**685-92.
15. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, et al. Intratumoral T Cells, Recurrence, and Survival in Epithelial Ovarian Cancer. *New England Journal of Medicine* 2003;**348:**203-13.
16. Clark CE, Hingorani SR, Mick R, Combs C, Tuveson DA, et al. Dynamics of the immune reaction to pancreatic cancer from inception to invasion. *Cancer Res* 2007;**67:**9518-27.
17. de Jong RA, Leffers N, Boezen HM, ten Hoor KA, van der Zee AGJ, et al. Presence of tumor-infiltrating lymphocytes is an independent prognostic factor in type I and II endometrial cancer. *Gynecologic Oncology* 2009;**114:**105-10.
18. Kondratiev S, Sabo E, Yakirevich E, Lavie O, Resnick MB. Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. *Clin Cancer Res* 2004;**10:**4450-6.
19. Ehrlich CE, Young PC, Stehman FB, Sutton GP, Alford WM. Steroid receptors and clinical outcome in patients with adenocarcinoma of the endometrium. *Am J Obstet Gynecol* 1988;**158:**796-807.
20. Jeon Y-T, Park I-A, Kim Y-B, Kim JW, Park N-H, et al. Steroid receptor expressions in endometrial cancer: Clinical significance and epidemiological implication. *Cancer Letters* 2006;**239:**198-204.
21. Hanekamp EE, Gielen SC, Smid-Koopman E, Kuhne LC, de Ruiter PE, et al. Consequences of loss of progesterone receptor expression in development of invasive endometrial cancer. *Clin Cancer Res* 2003;**9:**4190-9.
22. Kim YB, Holschneider CH, Ghosh K, Nieberg RK, Montz FJ. Progestin alone as primary treatment of endometrial carcinoma in premenopausal women. *Cancer* 1997;**79:**320-7.

23. Yahata T, Fujita K, Aoki Y, Tanaka K. Long-term conservative therapy for endometrial adenocarcinoma in young women. *Hum Reprod* 2006;**21:**1070-5.
24. Thigpen JT, Brady MF, Alvarez RD, Adelson MD, Homesley HD, et al. Oral medroxyprogesterone acetate in the treatment of advanced or recurrent endometrial carcinoma: a dose-response study by the Gynecologic Oncology Group. *J Clin Oncol* 1999;**17:**1736-44.
25. Wang Y, Hanifi-Moghaddam P, Hanekamp EE, Kloosterboer HJ, Franken P, et al. Progesterone inhibition of Wnt/beta-catenin signaling in normal endometrium and endometrial cancer. *Clin Cancer Res* 2009;**15:**5784-93.
26. Ward EC, Hoekstra AV, Blok LJ, Hanifi-Moghaddam P, Lurain JR, et al. The Regulation and Function of the Forkhead Transcription Factor, Forkhead Box O1, Is Dependent on the Progesterone Receptor in Endometrial Carcinoma. *Endocrinology* 2008;**149:**1942-50.
27. Scholten AN, Creutzberg CL, van den Broek L, Noordijk EM, Smit V. Nuclear β-catenin is a molecular feature of type I endometrial carcinoma. *The Journal of Pathology* 2003;**201:**460-5.
28. Tavassoli FA, Devilee P. *World Health Organisation: Tumours of the Breast and Female Genital Organs (Classification of Tumours).* 1 ed: IARCPress-WHO; 2003.
29. Pecorelli S. Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. *Int J Gynaecol Obstet* 2009;**105:**103-4.
30. Smid-Koopman E, Kuhne LC, Hanekamp EE, Gielen SC, De Ruiter PE, et al. Progesterone-induced inhibition of growth and differential regulation of gene expression in PRA- and/or PRB-expressing endometrial cancer cell lines. *J Soc Gynecol Investig* 2005;**12:**285-92.
31. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;**19:**185-93.
32. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proceedings of the National Academy of Sciences of the United States of America* 2001;**98:**5116-21.
33. Kim JJ, Chapman-Davis E. Role of progesterone in endometrial cancer. *Semin Reprod Med* 2010;**28:**81-90.
34. Mokhtar NM, Cheng CW, Cook E, Bielby H, Smith SK, et al. Progestin regulates chemokine (C-X-C motif) ligand 14 transcript level in human endometrium. *Mol Hum Reprod* 2010;**16:**170-7.
35. Bruner-Tran KL, Eisenberg E, Yeaman GR, Anderson TA, McBean J, et al. Steroid and Cytokine Regulation of Matrix Metalloproteinase Expression in Endometriosis and the Establishment of Experimental Endometriosis in Nude Mice. *J Clin Endocrinol Metab* 2002;**87:**4782-91.
36. Ace CI, Okulicz WC. Microarray profiling of progesterone-regulated endometrial genes during the rhesus monkey secretory phase. *Reprod Biol Endocrinol* 2004;**2:**54.
37. Thiery JP, Acloque H, Huang RYJ, Nieto MA. Epithelial-Mesenchymal Transitions in Development and Disease. *Cell* 2009;**139:**871-90.
38. Allavena P, Germano G, Marchesi F, Mantovani A. Chemokines in cancer related inflammation. *Experimental Cell Research*;**In Press, Accepted Manuscript**.
39. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* 2006;**147:**1097-121.
40. Nicholson RI, Gee JM, Knowlden J, McClelland R, Madden TA, et al. The biology of antihormone failure in breast cancer. *Breast Cancer Res Treat* 2003;**80 Suppl 1:**S29-34; discussion S5.
41. Avila DM, Zoppi S, McPhaul MJ. The androgen receptor (AR) in syndromes of androgen insensitivity and in prostate cancer. *J Steroid Biochem Mol Biol* 2001;**76:**135-42.
42. Hanekamp EE, Gielen SC, De Ruiter PE, Chadha-Ajwani S, Huikeshoven FJ, et al. Differences in invasive capacity of endometrial cancer cell lines expressing different progesterone receptor isotypes: possible involvement of cadherins. *J Soc Gynecol Investig* 2005;**12:**278-84.
43. Hanekamp EE, Kuhne EC, Smid-Koopman E, de Ruiter PE, Chadha-Ajwani S, et al. Loss of progesterone receptor may lead to an invasive phenotype in human endometrial cancer. *Eur J Cancer* 2002;**38 Suppl 6:**S71-2.
44. Yee DS, Tang Y, Li X, Liu Z, Guo Y, et al. The Wnt inhibitory factor 1 restoration in prostate cancer cells was associated with reduced tumor growth, decreased capacity of cell migration and invasion and a reversal of epithelial to mesenchymal transition. *Mol Cancer* 2010;**9:**162.

45. Brabletz T, Jung A, Hermann K, Gunther K, Hohenberger W, et al. Nuclear overexpression of the oncoprotein beta-catenin in colorectal cancer is localized predominantly at the invasion front. *Pathol Res Pract* 1998;**194:**701-4.

46. Naishiro Y, Yamada T, Takaoka AS, Hayashi R, Hasegawa F, et al. Restoration of epithelial cell polarity in a colorectal cancer cell line by suppression of beta-catenin/T-cell factor 4-mediated gene transactivation. *Cancer Res* 2001;**61:**2751-8.

47. Hu C, Xiong J, Zhang L, Huang B, Zhang Q, et al. PEG10 activation by co-stimulation of CXCR5 and CCR7 essentially contributes to resistance to apoptosis in CD19+CD34+ B cells from patients with B cell lineage acute and chronic lymphocytic leukemia. *Cell Mol Immunol* 2004;**1:**280-94.

48. Ip WK, Lai PB, Wong NL, Sy SM, Beheshti B, et al. Identification of PEG10 as a progression related biomarker for hepatocellular carcinoma. *Cancer Lett* 2007;**250:**284-91.

49. Jie X, Lang C, Jian Q, Chaoqun L, Dehua Y, et al. Androgen activates PEG10 to promote carcinogenesis in hepatic cancer cells. *Oncogene* 2007;**26:**5741-51.

50. Liu D-c, Yang Z-l, Jiang S. Identification of PEG10 and TSG101 as Carcinogenesis, Progression, and Poor-Prognosis Related Biomarkers for Gallbladder Adenocarcinoma. *Pathology & Oncology Research* 2011**:**1-8.

51. Wang X, Yuling H, Yanping J, Xinti T, Yaofang Y, et al. CCL19 and CXCL13 synergistically regulate interaction between B cell acute lymphocytic leukemia CD23+CD5+ B Cells and CD8+ T cells. *J Immunol* 2007;**179:**2880-8.

52. Kelly RW, Illingworth P, Baldie G, Leask R, Brouwer S, et al. Progesterone control of interleukin-8 production in endometrium and chorio-decidual cells underlines the role of the neutrophil in menstruation and parturition. *Hum Reprod* 1994;**9:**253-8.

53. Berry KK, Varney ML, Dave BJ, Bucana CD, Fidler IJ, et al. Expression of interleukin-8 in human metastatic endometrial carcinoma cells and its regulation by inflammatory cytokines. *Int J Gynecol Cancer* 2001;**11:**54-60.

54. Giangrande PH, McDonnell DP. The A and B isoforms of the human progesterone receptor: two functionally different transcription factors encoded by a single gene. *Recent Prog Horm Res* 1999;**54:**291-313; discussion -4.

55. Giangrande PH, Pollio G, McDonnell DP. Mapping and characterization of the functional domains responsible for the differential activity of the A and B isoforms of the human progesterone receptor. *J Biol Chem* 1997;**272:**32889-900.

56. Tetel MJ, Giangrande PH, Leonhardt SA, McDonnell DP, Edwards DP. Hormone-dependent interaction between the amino- and carboxyl-terminal domains of progesterone receptor in vitro and in vivo. *Mol Endocrinol* 1999;**13:**910-24.

57. Giangrande PH, Kimbrel EA, Edwards DP, McDonnell DP. The opposing transcriptional activities of the two isoforms of the human progesterone receptor are due to differential cofactor binding. *Mol Cell Biol* 2000;**20:**3102-15.

58. Yang L. TGFbeta, a potent regulator of tumor microenvironment and host immune response, implication for therapy. *Curr Mol Med* 2010;**10:**374-80.

59. Yang L, Pang Y, Moses HL. TGF-[beta] and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends in Immunology* 2010;**31:**220-7.

60. Pfeifer M, Schirmer U, Geismann C, Schafer H, Sebens S, et al. L1CAM expression in endometrial carcinomas is regulated by usage of two different promoter regions. *BMC Mol Biol* 2010;**11:**64.

61. Gavert N, Conacci-Sorrell M, Gast D, Schneider A, Altevogt P, et al. L1, a novel target of beta-catenin signaling, transforms cells and is expressed at the invasive front of colon cancers. *J Cell Biol* 2005;**168:**633-42.

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# Chapter 4

## Müllerian origin of ovarian cancer

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Each year, approximately 225,000 women worldwide are diagnosed with epithelial ovarian cancer, accounting for 3,7% of all women-related cancers. Although this incidence may seem relatively low, with more than 140.000 deaths each year, it is considered the most lethal gynecological malignancy[1]. High mortality is caused by the fact that by the time a patient experiences symptoms, the disease is usually spread-out in the abdomen. Furthermore, since in most patients microscopic disease is present after debulking surgery, chemotherapy is a crucial part of the treatment and even though initially most patients respond well, eventually chemoresistant disease will develop[2]. As a result, in the Netherlands overall 5-year-survival of ovarian cancer patients is approximately 41%, and in total almost 69% of patients die from the disease. Even more devastating, five-year survival from the most frequently diagnosed stage III or IV disease, is only a disappointing 28,6% and 14,1% respectively[3].
Delayed diagnosis is mainly caused by two important factors. Firstly, ovarian cancer shows late and unspecific symptoms such as fatigue, nausea, abdominal (pelvic) pain, bloating and feeling full, symptoms commonly present in many women and in many types of disease. Secondly, the origin of epithelial ovarian cancer is still debated amongst scientists and clinicians, making development of tools for early detection very difficult.

For many decades the ovarian surface epithelium (OSE) was appointed as the origin of epithelial ovarian cancer[2]. In the OSE model, ovarian carcinogenesis is thought to be triggered by reactive oxygen species and cytokine induced genotoxic damage of the OSE with each ovulation. Damaged OSE cells would invaginate into the ovarian stroma, thus forming so called cortical inclusion cysts (CICs). Through a process called metaplasia these cysts eventually obtain a Müllerian phenotype and under influence of locally produced high hormone levels, these cells eventually become malignant. However, this model needs revision because inclusion cysts were found to be similarly represented in both high risk patients and controls and equally important, precursor lesions of ovarian cancer in the OSE were never found[2, 4]. This hypothesis also suggests that ovarian cancer is better differentiated then its tissue of origin which goes against our current opinion on the development of cancer. Furthermore, although sometimes found as a cystic mass within the ovarian cortex, an important subset of serous ovarian carcinomas is found at the ovarian surface, frequently associated with serous tubal and peritoneal carcinoma. Knowing this, in 1999, Dubeau suggested that, because of the resemblance of ovarian cancer to Müllerian duct derived tissues, the role of components of the Müllerian system should be considered in ovarian carcinogenesis[5]. This hypothesis initiated a shift in paradigm about ovarian cancer and triggered many researchers and clinicians to search for an alternative origin for ovarian cancer. In this review we will describe clinical and more basic research that has been performed to reveal the origin of ovarian cancer and unravel the process of early carcinogenesis.

## 1. Paradigm shift: Identification of precursor lesions for serous ovarian cancer in the distal fallopian tube.

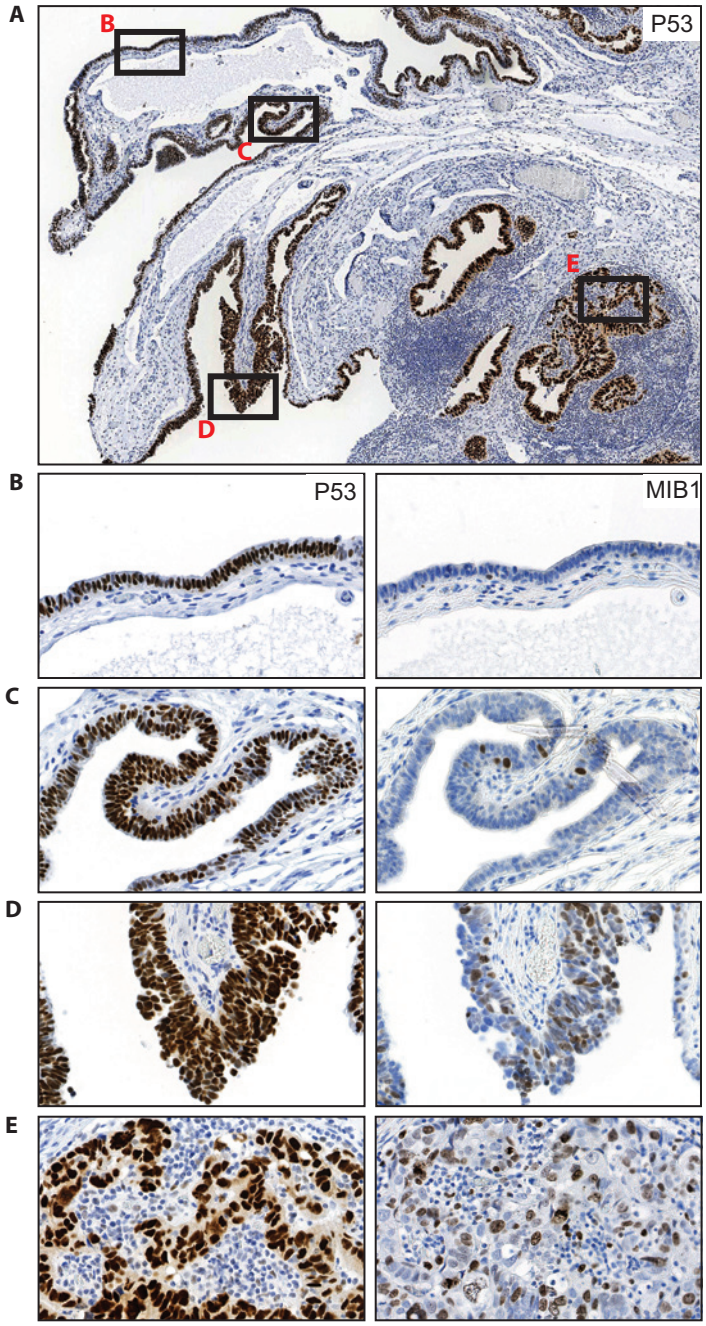
Triggered by reports on occult serous tumors in the fallopian tubes (oviducts) of women at risk for hereditary ovarian cancer (BRCA1 and 2 mutation carriers)[6-8], Piek et al., 2001, investigated the fallopian tubes of woman undergoing prophylactic bilateral salpingo-oophorectomy (pBSO) for a BRCA gene mutation[9]. Here, it was found that 50% of patients harbored regions of epithelial dysplasia in the distal fallopian tube epithelium, characterized by a shift towards the secretory phenotype with complete loss of ciliated cells and an increase of proliferative capacity, while no aberrations were found within the OSE[9]. Based on this and on the limitations of the existing hypothesis on serous ovarian carcinogenesis, a new hypothesis appointed the distal fallopian tube epithelium as the origin of serous ovarian cancer.

As more researchers investigated the fallopian tube epithelium as the site of origin for serous ovarian carcinoma, next to dysplasia, serous tubal intraepithelial carcinomas (STICs) were identified. Using a well thought-out protocol for examination of the fallopian tube, the SEE-FIM protocol, Meideros et al found STICs in 30,8% of women undergoing pBSO because of a BRCA gene mutation[10]. The presence of STICs was confirmed by many other research groups, although the high prevalence found by Medeiros et al. appears to be an exception and prevalence of STICs in pBSO patients usually varies between 1,0% and 12,0%[11-17]. STICs are characterized by intra-epithelial carcinoma in continuity with the normal mucosal epithelium, epithelial stratification, high nuclear to cytoplasmic ratio, nuclear atypia, loss of ciliated cells, high numbers of proliferating cells and mutations in the P53 tumor suppressor gene, characteristics also present in serous ovarian carcinoma[10]. STICs were also found to be present in the fallopian tube epithelium in as much as 45-60% of serous ovarian carcinoma patients[17-19]. These data suggest STICs to be a potential precursor lesion for serous ovarian cancer.

Next to malignant STICs, the presence of P53 signatures was described[10]. P53 signatures are regions that show strong p53-immunostaining but are non-proliferative and appear histopathologically benign[10]. The regions are composed of secretory cells that exhibit a serous phenotype and stain for γ-H2AX, a DNA-damage marker. P53 signatures occur both in BRCA gene mutation carriers and in controls, suggesting that the presence of these signatures is a normal phenomenon[10, 17]. However, P53 signatures were observed to be more frequently present in fallopian tubes containing STICs and were found in continuity with STICs[17, 20] (Fig. 1). Furthermore, the presence of γH2AX staining and abnormal P53 expression indicates that the tubal epithelium has experienced genotoxic damage, which can potentially trigger malignant transformation. Therefore, P53 signatures can be assessed as "benign" precursors of STICs, and subsequent serous ovarian cancer.

In confirmation of the relation between P53 signatures and STICs, morphological intermediates between p53 signatures and STICs were shown and identified as 'serous tubal intraepithelial lesions' (STILs)[20, 21].

**Figure 1: Continuous tubal precursor lesions in a patient with concurrent serous ovarian carcinoma.**




(A) In the fimbrial end of the fallopian tube of a serous ovarian carcinoma patient, P53 signatures (B), serous tubal intra-epithelial lesions (STILs)(C), serous tubal intra-epithelial carcinoma (STIC)(D) and tubal serous adenocarcinoma (E) are identified in continuum.

Additionally, identical P53 mutations were found in P53 signatures, STICs and concurrent serous carcinomas, making the hypothesis that STICs develop from 'precursor' p53 signatures and eventually spread to the ovaries a feasible one[20]. The suggestion that STICs may eventually disseminate to the ovaries was further strengthened by the finding of signs of possible epithelial-to-mesenchymal transition and the observation of P53 positive cells in abdominal washings of women with only a STIC at the fimbrial end of the distal oviduct[11, 17]. Furthermore, next to a shared P53 gene mutation, important molecular characteristics of serous ovarian cancer, such as expression of CA125, WT1, ER, PR, Vimentin, PAX2, PAX8 and HMGA2 were also found to be similar between STIC and concurrent ovarian cancer[17, 22-24]..

*In summary, malignant tubal precursor lesions for serous ovarian cancer (STICs) were identified in patients with a hierarchy in prevalence from patients susceptible for serous ovarian cancer, to patients with a concurrent serous ovarian cancer.*

## 2. Similarities between embryonic development of the Müllerian duct and the different ovarian cancer subtypes.

In the first paragraph we have indicated the fallopian tube as a possible site of origin of ovarian cancer, however, cells initiating the early malignant precursors of ovarian cancer in the fallopian tube are still unknown. In order to shed light on this issue, embryonic development will be discussed next.

The female reproductive tract stems from the intermediate mesoderm, and phenotypic development of the reproductive tract starts in the seventh week of development. Gonadal development is initiated a few weeks earlier, in the fifth week of pregnancy, in the caudal part of the ventromedial surface of the mesonephros and becomes prominent as the gonadal ridge protruding into the coelomic cavity. The gonads develop from migrating somatic cells, derived from the mesonephros, the surrounding mesenchymal and coelomic epithelium, and primordial germ cells migrating from the endodermal layer on the posterior wall of the yolk sac. During early development, the gonads are indifferent and development into male or female phenotype is depended on the presence of the SRY-gene on the Y-chromosome[25]. Under the influence of this gene, testes are formed, but in the absence of *SRY,* a gene called *DAX1* is continuously expressed, causing suppression of testis formation and allowing the gonads to develop into ovaries[26]. Development of the gonads into either the testes or ovaries, influences the development of the reproductive tract. The indifferent phase (bipotential stage) consists of the mesonephic (Wolffian) and the paramesonephic (Müllerian) ducts. If testes are present, Sertoli cells secrete testosterone and anti-Müllerian hormone (AMH), which causes the Wolffian duct to further develop and the Müllerian ducts to regress, respectively. If ovaries are present or if gonads are absent, testosterone and AMH are not secreted and the system differentiates into a female phenotype[27].

Even though the Müllerian duct and ovarian surface epithelium are both derived from the embryonic coelomic epithelium, the Müllerian duct stems from a specific subset of cells in the anterior region of the coelomic epithelium adjacent to the mesonephros. Müllerian duct development is initiated under the influence of *WNT4* secreted by the coelomic epithelium, by invagination of *LIM1* and *PAX2* expressing mesoepithelial cells creating a coelomic opening[28-31]. After invagination, the primitive Müllerian duct extends to and interacts with the still preexisting Wolffian duct. Under the influence of *WNT9B* expressing epithelial cells of the Wolffian duct, posterior elongation of the *LIM1* expressing epithelial cells is initiated and the Müllerian duct extends towards the cloaca[32]. Final outgrowth of the Müllerian duct epithelium is completed by widespread proliferation along the developing duct and at its growing tip and both of the Müllerian ducts fuse to form the uterovaginal tube[31, 33].
After initiation and posterior elongation of the Müllerian duct, posterior differentiation of the primitive Müllerian duct into vagina, cervix, uterus and oviducts depends on *WNT7A* expressed by oviductal and uterine epithelial cells and *WNT5A* expressed by uterine, cervical and vaginal mesenchymal cells[34, 35]. In addition to Wnt signaling, posterior differentiation of the Müllerian duct is also mediated by the actions of members of the *Hox* family of homeobox genes: *HOXA9* is expressed in the developing tubal epithelium, *HOXA10* in the developing uterus, *HOXA11* in the lower uterine segment and cervix and *HOXA13* in the upper part of the vagina[36]. Interestingly, maintenance of *HOXA10* and *HOXA11* expression is under the influence of *WNT5A* and *WNT7A*[34, 35].

Due to their involvement in Müllerian duct initiation and development, the role of *WNT* signaling, *HOX* genes and *PAX2* in ovarian carcinogenesis was studied. Although mainly investigated in endometrioid ovarian cancer, *WNT* signaling is an important factor in progression, survival and chemoresistance of serous ovarian cancer. High levels of *WNT5A* expression in serous ovarian cancer predict poor overall and progression-free survival[37]. Furthermore, *WNT5A* overexpression, induced in the human ovarian cancer cell line SKOV3, causes decreased chemosensitivity, which is in agreement with the earlier observed increased *WNT5A* expression in ovarian cancer cells with acquired oxaliplatin resistance[37, 38]. In contrast, *WNT5A* was also found to suppress growth of ovarian cancer cell lines by triggering cellular senescence[39]. Overexpression of *WNT7A* was found in invasive serous ovarian carcinoma and overexpression of *WNT7A* in OVCAR-3 and SKOV3 ovarian cancer cells promotes proliferation, migration and invasion[40]. Interestingly, *WNT7A* expression in adult life becomes restricted to epithelial cells of the oviduct and uterine luminal epithelium, but not in the ovary and the OSE[35, 40, 41]. Furthermore, *WNT9B* is highly expressed in ovarian cancer, but not the OSE[42].

Next to their function in Müllerian duct differentiation, the special restricted expression of *HOX* genes continues to be present throughout adult life and is thought to be crucial for maintaining the epithelial plasticity necessary for functional changes which occur during menstruation and ovulation[43]. This finding is of interest, because the major subtypes of epithelial ovarian cancer are

distinguished by their morphological resemblance to the specialized epithelia of the reproductive tract that have been derived from the Müllerian duct. Serous ovarian cancer is typically papillary or cystic and resembles the epithelium of the fallopian tube. In contrast, endometrioid and mucinous ovarian cancer resemble the endometrial-like glands and endocervical epithelium, respectively[2]. Because of this resemblance and because the expression of *HOX* genes is confined to specific parts of the Müllerian derived epithelium, the expression of *HOX* genes in epithelial ovarian cancer was investigated. Interestingly, overexpression of specifically *HOXA9, HOXA10, HOXA11* was shown in serous, endometrioid and mucinous ovarian carcinoma, respectively[44]. These findings are of interest because this expression pattern coincides with the physiological expression pattern of these HOX genes: *HOXA9* is expressed in the fallopian tube, *HOXA10* in the endometrium and *HOXA11* in the endocervix. Importantly, *HOXA9, HOXA10* and *HOXA11* are not expressed in the ovarian surface epithelium[44].

Finally, *PAX2* is coexpressed with *LIM1* by cells in the earliest anlage of the Müllerian duct (Kobayashi 2003). Interestingly, *PAX2* was found to be expressed in ovarian papillary serous carcinomas, the epithelium of the fallopian tube, endometrium and endocervix, but not in the OSE, ovarian surface epithelium derived inclusion cysts and the ovary itself[23]. In contrast, Ozcan et al., 2001, did show focal PAX2 expression in the OSE, next to high expression within the fallopian tube and epithelial ovarian cancer[45]. However, since PAX2 expressing cells initiate Müllerian duct invagination from the coelomic epithelium and number of rudimentary Müllerian cells in proximity of this area might cause focal OSE expression.

*In summary, many similarities and shared characteristics have been identified between early development of the various M*üllerian duct *derived organs and the different epithelial ovarian cancer subtypes.*

## 3. The identification of stem cells that could be involved in initiation of ovarian cancer.

There is tentative evidence to postulate that at least in a number of cases a genetically changed stem cell is the initiating event in malignant transformation[46, 47]. Therefore, investigations into the identification of stem cells that could be involved in ovarian carcinogenesis are important.

In 2008, using doxycycline inducible histone2B-GFP and BrdU pulse-chase experiments, Szotek et al., identified a population of long term label-retaining cells (3 months of chase) in the ovarian surface epithelium of adult mice as potential stem or progenitor cells[48]. Label-retaining cells were slow cycling and showed asymmetric division. Furthermore, label-retaining cells showed a functional proliferative response to estrogen exposure in vivo and enhanced colony formation in vitro. However, no evidence of self-renewal, a main characteristic of stem cells, was found [46]. Next to this, the capacity of identified label retaining cells upon mutation to induce ovarian cancer was

not addressed and other regions surrounding the ovaries, such as the fallopian tube, as a putative source of stem or progenitor cells were not assessed.

In a subsequent effort to investigate the origin of ovarian cancer in mice, a localized pool of stem-like cells was found to be clustered in the ovarian hilum region, the transitional area which forms the junction between the OSE, mesothelial peritoneum and tubal epithelium[49]. Cells were identified using BrdU pulse chase experiments and immunohistochemical analysis for *Aldh1*. Microdisected ovarian hilum cells were slow cycling, formed larger colonies, developed more spheroids and could be propagated longer as compared to normal OSE cells. Furthermore, using FACsorting, *Aldh1* expressing OSE cells were isolated and were shown to express stem cell markers *Aldh1*, *CD133*, *Ck6b*, *Lgr5* and *Lef1*. In order assess the malignant potential of ovarian hilum cells, adenoviral delivery of C-recombinase in the ovarian bursa of *Trp53*$^{loxp/loxp}$; *Rb1*$^{loxp/loxp}$ animals was accomplished, resulting in early neoplastic lesions in the hilum. Additionally, Trp53 and Rb1-deficient primary cultured hilum and OSE cells were transplanted intraperitoneally. Upon transplantation, 7/8 mice injected with hilum cells developed high grade serous adenocarcinomas with metastasis to the lung, while only 1/12 mice injected with OSE cells developed a non-metastatic carcinoma. The results of this study led to the postulation that the transitional zone between OSE, mesothelial peritoneum and tubal epithelium, harbors a stem cell niche, which, when it becomes mutated, has the potential to give rise to serous ovarian cancer.

Also using the doxycycline inducible histone2B-GFP model, Wang et al (2012) identified a population of long term label-retaining cells (12 weeks of chase) in the distal and fimbrial part of the fallopian tube[50]. These cells could, after FACsorting, form spheroids capable of self-renewal and upon serum stimulation (differentiation) these spheroids formed glandular structures, which expressed markers of mature Müllerian epithelial cells (ERα, PRab, Paep and Cd44). In addition, in this study, no label-retaining cells were found to be present within the OSE, while label-retaining cells were present in the distal oviduct up to 47 weeks of chase. The presence of these stem-like cells in the distal and fimbrial part of the fallopian tube is of interest, because their location coincides with the earliest anlage of the Müllerian duct during embryonic development. Interestingly, the distal fallopian tube contains a segment that is in continuity with the ovarian hilum and pelvic mesothelium, forming a Müllerian-mesothelial (mesoepihtelial) junction. Therefore the stem-like cells identified in the ovarian hilum might be interrelated with stem-like cells identified in the distal oviduct (Flesken-Nikitin, Wang). In addition to this, 80-93% of tubal precursors of ovarian cancer are identified within the distal oviduct[17, 19].

Because endometrial intra-epithelial carcinoma (EIC) is also hypothesized to be a precursor lesion of serous ovarian cancer, a potential role for endometrial stem cells in ovarian carcinogenesis was proposed[51]. The first evidence of an endometrial stem cell was obtained by plating out purified single cell suspensions of endometrial epithelial and stromal cells, which showed 0,22% of epithelial and 1,25% of stromal cells to be able to form large colonies, which could be replated several times[52]. This clonal capacity was confirmed by a number of research groups[53-55] and when

grown in Matrigel, Gargett et al. (2009) demonstrated that a single colony forming epithelial cell was able to form large cytokeratin expressing gland-like structures[56]. Furthermore, putative endometrial stromal stem cells were shown to be able to differentiate in multiple mesenchymal lineages and even into functional epithelium[56-59]. However, in all studies, a single and specific stem cell was not identified nor isolated. Using BrdU labeling, Chan et al., (2006) showed label retaining cells (LRCs) to be present in the luminal epithelium at 8 weeks of chase[60] and in the stromal endometrial-myometrial junction at 12 weeks of chase. The presence of BrdU-LRCs in both the endometrial epithelium and stroma, was confirmed by Cervelló et al. (2007) and here, LRCs were found to co-localize with stem cell markers c-KIT and POU5F1/OCT-4[61]. Unfortunately, in both studies, stem cell characteristics of the LRCs, such as self-renewal, spheroid forming capacity and growth in recipient animals were not addressed. Wang et al. (2012) confirmed the presence of LRCs in the endometrium, using doxycycline H2B-GPF pulse-chase labeling, and found LRCs to be present up until 4 and 12 weeks in epithelial and stromal endometrial cells respectively[50]. Interestingly, as described earlier, LRCs were identified in the distal fallopian tube up to 1 year after pulse and showed stem-like characteristics. Other investigations on the presence of endometrial stem cells showed that, donor-derived bone marrow cells were identified in the endometrium of patients receiving bone marrow transplantation[62]. Lethally irradiated female mice, in which LacZ-expressing bone marrow cells of a male donor were identified in the epithelium of the endometrium and peritoneal endometriosis, further confirmed the potential of bone marrow cells as stem cells of the endometrium[63].

*Summarizing, progenitor or stem-like cells were identified in the OSE, ovarian hilum, fallopian tube and endometrium. However, their true potential in ovarian cancer initiation is still to be determined.*

## 4. Ovarian cancer initiation in mouse models.

Ovarian cancer cell lines and xenografts have been used extensively over the last decades and proved effective to investigate chemoresistance, molecular mechanisms of action and biological behavior of epithelial ovarian cancer[64]. However, cell lines and xenograft models have their limitation and animal models mimicking initiation, early development and metastatic spread of epithelial ovarian cancer are rare. Therefore, models in which genes are conditionally knocked in or out have been developed for epithelial ovarian cancer. Below we will discuss the most important models and review what data are presented that add to the discussion on the origin of ovarian cancer.

Adenoviral delivery of C-recombinase (Ad-Cre) has been extensively used as a tool to induce recombination in tissues inside the bursal pouch surrounding the ovary and distal oviduct in mice. In order to assess genes frequently involved in ovarian carcinogenesis, bursal injection of adenoviral-Cre (AdCMV-Cre) in *P53*$^{lox/lox}$;*Rb1*$^{lox/lox}$ animals was used[65]. Recombination of *P53* and *Rb1* resulted in ovarian epithelial cancer in 97% of animals, with ascites (24%) and metastasis spread to the contralateral ovary (15%), lung (18%) and liver (6%). Control experiments indicated that Ad-

Cre administration resulted in recombinase activity in the OSE cells. Furthermore, OSE cells with conditional deletions of *P53* and *Rb1* displayed an increased proliferative activity[65]. Importantly, injection of Ad-Cre into the bursal cavity also delivers Ad-Cre to the fimbrial and distal part of the oviduct. However, possible recombination and involvement of Müllerian duct derived tissues as an origin of epithelial ovarian cancer in this study was not discussed.

Simultaneously, Dinulescu et al. (2005) used bursal delivered Ad-Cre to induce recombination in *Pten*$^{lox/lox}$;*Isl-Kras*$^{G12D/+}$ animals and found rapidly developing, widely metastatic, endometrioid ovarian adenocarcinomas in 100% of animals, only 7 weeks after delivery[66]. Interestingly, animals which were recombined for *Kras*$^{G12D}$ alone, only showed ovarian endometriosis, which is associated with endometrioid ovarian carcinogenesis[66-68]. Importantly, Cre-activity in these animals was confirmed in OSE cells, but was also documented in the bursa and the distal oviduct.

Wu et al (2007) reviewed 72 ovarian endometrioid adenocarcinoma tissues and observed defects in the *PI3K/Pten* and Wnt/β-catenin signaling pathways in a subset of these tumors[69]. Based on this, Ad-Cre injection into the bursa was used to recombine *Apc*$^{lox/lox}$ and *Pten*$^{lox/lox}$. Here, adenocarcinomas developed which were morphologically similar to human ovarian endometrioid adenocarcinoma in 100% of animals. Furthermore, 76% of mice developed hemorrhagic ascites and 21% developed overt peritoneal dissemination[69]. Even though whole organ staining for Adenoviral-Cre revealed recombinase activity in OSE cells, the authors were inconclusive for Cre activity in the distal oviduct.

Using Adenoviral-GFP and Adenoviral-LacZ as controls, Clark-Knowles et al. (2007) showed infection to be seemingly confined to the OSE cells (no expression in ovarian fatpad, oviduct and uterus)[70]. Ad-Cre delivery to *Brca1*$^{lox/lox}$ animals resulted in increased accumulation of premalignant changes (hyperplasia, a 4-fold increase in epithelial invaginations and inclusion cysts), while Ad-Cre delivery to *P53*$^{lox/lox}$ animals resulted in tumors in 100% of animals and tumor progression was accelerated in *P53*$^{lox/lox}$;*Brca1*$^{lox/lox}$ mice[70]. Interestingly, the induced tumors were classified as leiomyosarcomas, which the authors themselves suggested to have arisen from the ovarian bursa and not from OSE cells or distal oviduct. Kim at al., 2010 performed similar experiments using Adenoviral-Cre, and was able to show increased proliferation of OSE from *Brca1*$^{lox/lox}$ and *Brca2*$^{lox/lox}$;*P53*$^{lox/lox}$ recombined mice[71]. However and surprisingly, no evidence of involvement of recombined *Brca1*$^{lox/lox}$, *Brca2*$^{lox/lox}$ or *P53*$^{lox/lox}$ in ovarian carcinogenesis was shown.

Finally, Laviolett et al. (2010) induced recombination of tgCAG-LS-Tag (resulting in a functional SV40 Tag) by bursal injection of Ad-Cre and these mice developed poorly differentiated ovarian tumors, with metastasis in the pancreas and spleen[72]. However, the distal oviduct and fimbriae were not assessed in these investigations.

Even though in many models epithelial ovarian cancer growth was established, adenoviral-Cre injections into the ovarian bursa will not only recombine the affected (loxed) genes in the OSE cells, but will also cause Cre-meditated recombination in cells of the fimbriae and distal oviduct. Therefore, using this technique it is not possible to discriminate between OSE cells and cells located in the fimbrial region of the distal oviduct as the origin of ovarian carcinogenesis.

In order to use a more targeted approach, Connolly et al., 2003, used the *AmhR2* (*MISIIR*) promoter to drive SV40 TAg[73]. Here, poorly differentiated serous ovarian cancer was observed in 50% of all animals. Next to these ovarian tumors, intraperitoneal ascites and peritoneal implants were observed. Immunohistological staining to detect *SV40-TAg* revealed expression in OSE cells, but also in patches of epithelial cells in the oviduct and uterus. Furthermore, using PCR, *AmhR2* was shown to be expressed in the ovary as well at low levels in the oviduct and uterus. In contrast, transgenic mice in which the *AmhR2* promoter was used to drive *PIK3CA* expression and activity (a much weaker oncogenic signal), only showed hyperplasia of the OSE[74].

In mice in which AmhR2-Cre was used to drive recombination of *Pten*$^{lox/lox}$;*Isl-Kras*$^{G12D}$, low-grade ovarian serous papillary adenocarcinomas were formed in 100% of mice[75, 76]. Interestingly, isolated recombined OSE cells displayed a temporal change in expression of Müllerian epithelial markers, grew in soft agar and developed ectopic invasive tumors in recipient mice[76]. The Müllerian duct as a possible site of origin of ovarian cancer, however, was neither reviewed, nor discussed in relation to these experiments[75, 76].

Using *AmhR2*-Cre, *Dicer*, an essential gene for micro RNA synthesis, and *Pten*, a key tumor suppressor inhibiting the *PI3K* pathway were conditionally deleted[77]. As a result, high-grade serous carcinomas arising from the fallopian tube with spread to the ovary and metastasis throughout the abdominal cavity were identified in 100% of mice and closely resembled human serous cancer. Interestingly, removal of the oviducts at an early age prevented cancer formation. However, it is important to note that so far there has not been a role for Dicer in ovarian carcinogenesis and, furthermore, using this model, cancer initiation seems to start from stromal cells of the oviduct while in humans tubal precursors of serous ovarian cancer are epithelial.

Tanwar et al., 2012 combined *AmhR2*-Cre with *Apc*$^{lox/lox}$ and observed development of epithelial inclusion cysts and, in much older animals, high grade ovarian endometrioid adenocarcinoma[78]. The finding of endometrioid ovarian cancer is in agreement with observations that in this subtype, Wnt/β-catenin signaling is often activated[79].

In an effort to prove the Müllerian origin of endometrioid ovarian cancer, *Pgr*-Cre induced conditional recombination of *Apc*$^{lox/lox}$ in the oviduct was used[80]. Interestingly, in this model the OSE cells are not affected. As described before, Wnt signaling is an important oncologic factor in human endometrioid ovarian cancer and *APC* mutations are frequently found[79]. Interestingly, in this model, tubal intra-epithelial carcinomas developed, starting from 10 weeks of age, which show high resemblance to human tubal intra-epithelial carcinomas. With age, these TICs were shown to evolve and developed into endometrioid tubal and ovarian tumors resembling human endometrioid tubal and ovarian cancer growth. Next to these tubal and ovarian tumors, loco-regional spread to the utero-ovarian ligament was shown[80].

Additionally models, in which not the OSE cells or Müllerian duct but the granulosa cells were targeted, also need to be discussed[81-83]. Chen et al., described early alterations in OSE cells in FshR-knockout animals, eventually resulting in serous papillary adenoma of the ovaries[81]. Another

model used FshR-Cre to target Brca1$^{lox/lox}$ in granulosa cells of the ovary. In these animals, grossly visible serous cystadenomas were attached to the ovary, within the wall of the uterus, or on the external surface of the uterine horns. Interestingly, in these cystadenomas the Brca1 gene was not recombined indicating that factors secreted by the granulosa cells must have influenced tumorigenesis indirectly[82]. The finding that the uterine horns are also involved next to the ovaries is in line with the finding of tubal intraepithelial lesions in asymptomatic carriers of BRCA1 mutations[10, 15, 16] and seems to point to an extraovarian origin of ovarian cancer.

*In summary, some mouse models point towards the OSE cells and others to Müllerian duct derived tissues as the origin of epithelial ovarian cancer, but in essence none of these models are specific enough to provide a definitive answer to the question whether it are mutated or modified OSE cells, or cells from Müllerian origin that develop into the earliest malignant precursors of ovarian cancer.*

## 5. The secondary Müllerian system as a source of ovarian carcinogenesis.

In 1999, Dubeau suggested the secondary Müllerian system as a possible origin of epithelial ovarian cancer. The secondary Müllerian system consists of microscopic structures lined with Müllerian epithelium, commonly present in the paratubal and paraovarian areas, the ovarian medulla near the hilum and the deeper portions of the ovarian cortex[84]. These structures might be rudimentary remnants from the developing Müllerian duct but also include endosalphyngiosis (cysts lined with tubal epthelium), endocervicosis (cysts lined with endocervical epithelium) and endometriosis (functional endometrial-like tissue outside the uterus)[5, 84]. Interestingly, these structures can develop into large extra- or intra-ovarian cysts which share morphological characteristics with serous, mucinous or endometrioid ovarian cancer.

Endometriosis affects 5- 10% of woman of reproductive age and is therefore considered as a major gynecological health problem[85]. As in ovarian cancer, the origin of endometriosis is not clear but the most prevalent hypothesis is that endometrial stem cells appear in the abdominal cavity where they attach and migrate into surrounding tissues and organs[86]. Interestingly, endometrioid ovarian cancer also resembles the endometrium and recent investigations have indicated an association between endometriosis and endometrioid ovarian cancer[67, 68]. A strong increased risk for ovarian malignancies in women with endometriosis was identified in a large pooled case-control study where a significant association was found between history of self-reported endometriosis and clear-cell, low-grade serous and endometrioid ovarian cancer[67]. Furthermore, similar gene mutations in ARID1a in endometrioid ovarian cancer and neighboring atypical endometriosis were found, indicating a genetical association between the two diseases[68]. The epidemiological relationship between endometriosis and ovarian cancer was further confirmed by Buis et al., 2013, who found an increased ovarian cancer risk in subfertile women with surgically diagnosed endometrioisis (REF). In addition to endometriosis, serous borderline tumors also were found in foci of endosalpingiosis in pelvic and para-aortic lymph nodes[87].

Furthermore, it was suggested that the rete ovarii, which consist of coiled microscopic ducts near the ovarian hilum, are part of the secondary Müllerian system. Interestingly, in some rodents, although rarely diagnosed, epithelial ovarian cancer seems to naturally arise from a dilatation of these rete ovarii[88, 89].
These findings are of interest because stem-like cells were identified in the ovarian hilum and therefore an association with lesions located in the ovarian hilum, such as rete ovarri, endosalpingiosis and endometriosis may be hypothesized[49].

*Even though most research is focused on either the fallopian tube or OSE as the origin of ovarian cancer, important findings appoint a role for other Müllerian duct derived structures such as the secondary Müllerian system in epithelial ovarian carcinogenesis.*

## 6. Not the OSE but tissues derived from the Müllerian duct are the origin of epithelial ovarian cancer: conclusions and future perspectives

Cortical inclusion cysts (CICs), derived from either the Müllerian duct or OSE, have been appointed as the origin of epithelial ovarian cancer[5, 90-92]. Even though some CICs appear mesothelial (OSE), most CICs resemble a Müllerian morphology[4, 93-95]. The OSE hypothesis corrects for this Müllerian appearance, by stating that stem or progenitor cells from the OSE acquire genetic modifications and regain Müllerian characteristics through metaplasia[90]. Ovarian cancer is induced by additional genetic disturbances and stimuli from the surrounding microenvironment, leading to dysplasia of the metaplastic CIC and culminating as full-scale epithelial ovarian cancer. If we, however, summarize all supporting and opposing arguments for either the OSE or the Müllerian duct as the origin of ovarian cancer the balance tips towards a Müllerian origin of ovarian cancer.

Scientific evidence supporting an OSE origin of ovarian cancer:
- OSE lined CICs have been described[4, 90, 93, 95],
- OSE cells and the Müllerian duct are both derived from a shared embryonic precursor, indicating that metaplasia to Müllerian duct like cells may be possible,
- From all cells present in the ovary, OSE cells are the only cell type for which metaplasia is feasible,
- Stem-like cell characteristics have been described for OSE cells[48, 96, 97],
- Atypical OSE cells were found directly adjacent serous ovarian cancer[98],
- Isolated mutated OSE cells, when transplanted in recipient mice, can show serous ovarian cancer growth[49].

Scientific evidence supporting a Müllerian duct origin of ovarian cancer:
- The three most important epithelial ovarian cancer subtypes represent Müllerian duct derived tissues[92],
- Genes important for Müllerian duct development and maintenance are highly expressed in ovarian cancer, but are not expressed in the OSE[23, 37-42, 44, 45, 79],

- Stem-like cells forming spheroids and capable of self-renewal were identified in the distal oviduct[50, 60, 61],
- Müllerian duct derived structures are found in the ovarian hilum and are possibly associated with stem or progenitor cells found in the ovarian hilum[49, 84],
- Mouse models in which the Müllerian duct is mutated, but not the OSE, show serous and endometrioid ovarian cancer[77, 80],
- Components of the secondary Müllerian system, such as endometriosis, endosalpingiosis, endocervicosis and rete ovarii, are found in the ovary and are associated with epithelial ovarian cancer[5, 67, 68, 84, 87-89],
- Most CICs are lined with Müllerian epithelium, and P53 expressing, dysplastic cells are found within CICs lined with Müllerian epithelium[94],
- Early benign (P53 signatures), intermediate (STILs) and clearly malignant (STICs) precursors of high grade serous ovarian cancer (all lesions of the distal oviduct) were identified with a hierarchy in prevalence from control, to patients at risk, to patients with a concurrent serous ovarian cancer[9-21].
- STICs are only identified in patients at risk or with a concurrent serous ovarian cancer[9-21],
- P53 signatures, STILs and STICs share identical P53 mutations with the concurrent serous ovarian cancer[20],
- In patients with pelvic serous carcinoma, which is indistinguishable from serous ovarian carcinoma, STICs are found but no ovarian lesions[19]. Furthermore, STICs and concurrent pelvic serous carcinoma display similar P53 mutations.

Upon reviewing these data, we appoint two possible mechanisms in which epithelial ovarian cancer is initiated based on the histopathological model of Kurman and Shih(2008)[99].
First, since type I ovarian tumors are typically ovarian confined and develop from borderline precursors, we hypothesize that these are derived from the oviduct or components of the secondary Müllerian system, such as ovarian endosalpingiosis or endometriosis, which over time have acquired further genetical disturbances due to ovulation-induced distress or stimuli from the ovarian stroma.
Second, type II high grade serous ovarian carcinomas are mainly confined to the ovary and are characterized by mutation of *TP53*. Therefore we hypothesize that ovulation-induced damage to the distal fallopian tube epithelium results in areas mutated for *TP53* (P53 signatures). Upon further genetic damage and increased proliferation, P53 signatures develop into STILs, which, progress to become STICs. When transformed, malignant STIC cells can exfoliate and, in addition to other peritoneal sites, implant on or in the ovary. As a result, type II high grade serous ovarian cancer develops.

In conclusion, there is abundant evidence that not the OSE but the Müllerian duct should be appointed as the origin of epithelial ovarian cancer and research aiming to unravel the earliest carcinogenic changes in Müllerian derived tissues is key to facilitate early detection and targeted therapy for ovarian cancer.

## References


1. Ferlay J SH, Bray F, Forman D, Mathers C and Parkin DM. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. [cited; Available from: http://globocan.iarc.fr, accessed on day/month/year.
2. Cannistra SA. Cancer of the Ovary. *New England Journal of Medicine* 2004;**351:**2519-29.
3. van Altena AM, Karim-Kos HE, de Vries E, Kruitwagen RF, Massuger LF, et al. Trends in therapy and survival of advanced stage epithelial ovarian cancer patients in the Netherlands. *Gynecol Oncol* 2012;**125:**649-54.
4. Stratton JF, Buckley CH, Lowe D, Ponder BA. Comparison of prophylactic oophorectomy specimens from carriers and noncarriers of a BRCA1 or BRCA2 gene mutation. United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Familial Ovarian Cancer Study Group. *J Natl Cancer Inst* 1999;**91:**626-8.
5. Dubeau L. The cell of origin of ovarian epithelial tumors and the ovarian surface epithelium dogma: does the emperor have no clothes? *Gynecol Oncol* 1999;**72:**437-42.
6. Rose PG, Shrigley R, Wiesner GL. Germline BRCA2 mutation in a patient with fallopian tube carcinoma: a case report. *Gynecol Oncol* 2000;**77:**319-20.
7. Zweemer RP, van Diest PJ, Verheijen RH, Ryan A, Gille JJ, et al. Molecular evidence linking primary cancer of the fallopian tube to BRCA1 germline mutations. *Gynecol Oncol* 2000;**76:**45-50.
8. Colgan TJ, Murphy J, Cole DE, Narod S, Rosen B. Occult carcinoma in prophylactic oophorectomy specimens: prevalence and association with BRCA germline mutation status. *Am J Surg Pathol* 2001;**25:**1283-9.
9. Piek JM, van Diest PJ, Zweemer RP, Jansen JW, Poort-Keesom RJ, et al. Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer. *J Pathol* 2001;**195:**451-6.
10. Medeiros F, Muto MG, Lee Y, Elvin JA, Callahan MJ, et al. The tubal fimbria is a preferred site for early adenocarcinoma in women with familial ovarian cancer syndrome. *Am J Surg Pathol* 2006;**30:**230-6.
11. Callahan MJ, Crum CP, Medeiros F, Kindelberger DW, Elvin JA, et al. Primary fallopian tube malignancies in BRCA-positive women undergoing surgery for ovarian cancer risk reduction. *J Clin Oncol* 2007;**25:**3985-90.
12. Carcangiu ML, Peissel B, Pasini B, Spatti G, Radice P, et al. Incidental carcinomas in prophylactic specimens in BRCA1 and BRCA2 germ-line mutation carriers, with emphasis on fallopian tube lesions: report of 6 cases and review of the literature. *Am J Surg Pathol* 2006;**30:**1222-30.
13. Hirst JE, Gard GB, McIllroy K, Nevell D, Field M. High rates of occult fallopian tube cancer diagnosed at prophylactic bilateral salpingo-oophorectomy. *Int J Gynecol Cancer* 2009;**19:**826-9.
14. Manchanda R, Abdelraheim A, Johnson M, Rosenthal AN, Benjamin E, et al. Outcome of risk-reducing salpingo-oophorectomy in BRCA carriers and women of unknown mutation status. *BJOG* 2011;**118:**814-24.
15. Mingels MJ, Roelofsen T, van der Laak JA, de Hullu JA, van Ham MA, et al. Tubal epithelial lesions in salpingo-oophorectomy specimens of BRCA-mutation carriers and controls. *Gynecol Oncol* 2012;**127:**88-93.
16. Reitsma W, de Bock GH, Oosterwijk JC, Bart J, Hollema H, et al. Support of the 'fallopian tube hypothesis' in a prospective series of risk-reducing salpingo-oophorectomy specimens. *Eur J Cancer* 2013;**49:**132-41.
17. Van der Horst PH, Wijnhoven RKE, Daal S, Mouthaan MH, Heijmans-Antonissen C, et al. Malignant transition of tubal precursors into serous ovarian cancer. *In submission* 2013.
18. Roh MH, Kindelberger D, Crum CP. Serous tubal intraepithelial carcinoma and the dominant ovarian mass: clues to serous tumor origin? *Am J Surg Pathol* 2009;**33:**376-83.
19. Kindelberger DW, Lee Y, Miron A, Hirsch MS, Feltmate C, et al. Intraepithelial carcinoma of the fimbria and pelvic serous carcinoma: Evidence for a causal relationship. *Am J Surg Pathol* 2007;**31:**161-9.
20. Lee Y, Miron A, Drapkin R, Nucci MR, Medeiros F, et al. A candidate precursor to serous carcinoma that originates in the distal fallopian tube. *J Pathol* 2007;**211:**26-35.
21. Vang R, Visvanathan K, Gross A, Maambo E, Gupta M, et al. Validation of an algorithm for the diagnosis of serous tubal intraepithelial carcinoma. *Int J Gynecol Pathol* 2012;**31:**243-53.
22. Jarboe EA, Miron A, Carlson JW, Hirsch MS, Kindelberger D, et al. Coexisting intraepithelial serous carcinomas of the endometrium and fallopian tube: frequency and potential significance. *Int J Gynecol Pathol* 2009;**28:**308-15.
23. Tong GX, Chiriboga L, Hamele-Bena D, Borczuk AC. Expression of PAX2 in papillary serous carcinoma of the ovary: immunohistochemical evidence of fallopian tube or secondary Mullerian system origin? *Mod Pathol* 2007;**20:**856-63.
24. Wei JJ, Wu J, Luan C, Yeldandi A, Lee P, et al. HMGA2: a potential biomarker complement to P53 for detection of early-stage high-grade papillary serous carcinoma in fallopian tubes. *Am J Surg Pathol* 2010;**34:**18-26.

25. Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. *Physiol Rev* 2007;**87:**1-28.
26. Ikeda Y, Takeda Y, Shikayama T, Mukai T, Hisano S, et al. Comparative localization of Dax-1 and Ad4BP/SF-1 during development of the hypothalamic-pituitary-gonadal axis suggests their closely related and distinct functions. *Dev Dyn* 2001;**220:**363-76.
27. Acien P. Embryological observations on the female genital tract. *Hum Reprod* 1992;**7:**437-45.
28. Masse J, Watrin T, Laurent A, Deschamps S, Guerrier D, et al. The developing female genital tract: from genetics to epigenetics. *Int J Dev Biol* 2009;**53:**411-24.
29. Kobayashi A, Shawlot W, Kania A, Behringer RR. Requirement of Lim1 for female reproductive tract development. *Development* 2004;**131:**539-49.
30. van der Horst PH, Wang Y, van der Zee M, Burger CW, Blok LJ. Interaction between sex hormones and WNT/beta-catenin signal transduction in endometrial physiology and disease. *Mol Cell Endocrinol* 2012;**358:**176-84.
31. Guioli S, Sekido R, Lovell-Badge R. The origin of the Mullerian duct in chick and mouse. *Dev Biol* 2007;**302:**389-98.
32. Carroll TJ, Park JS, Hayashi S, Majumdar A, McMahon AP. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev Cell* 2005;**9:**283-92.
33. Orvis GD, Behringer RR. Cellular mechanisms of Mullerian duct formation in the mouse. *Dev Biol* 2007;**306:**493-504.
34. Mericskay M, Kitajewski J, Sassoon D. Wnt5a is required for proper epithelial-mesenchymal interactions in the uterus. *Development* 2004;**131:**2061-72.
35. Miller C, Sassoon DA. Wnt-7a maintains appropriate uterine patterning during the development of the mouse female reproductive tract. *Development* 1998;**125:**3201-11.
36. Podlasek C, Houston J, McKenna KE, McVary KT. Posterior Hox gene expression in developing genitalia. *Evol Dev* 2002;**4:**142-63.
37. Peng C, Zhang X, Yu H, Wu D, Zheng J. Wnt5a as a predictor in poor clinical outcome of patients and a mediator in chemoresistance of ovarian cancer. *Int J Gynecol Cancer* 2011;**21:**280-8.
38. Varma RR, Hector SM, Clark K, Greco WR, Hawthorn L, et al. Gene expression profiling of a clonal isolate of oxaliplatin-resistant ovarian carcinoma cell line A2780/C10. *Oncol Rep* 2005;**14:**925-32.
39. Bitler BG, Nicodemus JP, Li H, Cai Q, Wu H, et al. Wnt5a suppresses epithelial ovarian cancer by promoting cellular senescence. *Cancer Res* 2011;**71:**6184-94.
40. Merritt MA, Parsons PG, Newton TR, Martyn AC, Webb PM, et al. Expression profiling identifies genes involved in neoplastic transformation of serous ovarian cancer. *BMC Cancer* 2009;**9:**378.
41. Yoshioka S, King ML, Ran S, Okuda H, MacLean JA, 2nd, et al. WNT7A regulates tumor growth and progression in ovarian cancer through the WNT/beta-catenin pathway. *Mol Cancer Res* 2012;**10:**469-82.
42. Steg A, Wang W, Blanquicett C, Grunda JM, Eltoum IA, et al. Multiple gene expression analyses in paraffin-embedded tissues by TaqMan low-density array: Application to hedgehog and Wnt pathway analysis in ovarian endometrioid adenocarcinoma. *J Mol Diagn* 2006;**8:**76-83.
43. Taylor HS, Vanden Heuvel GB, Igarashi P. A conserved Hox axis in the mouse and human female reproductive system: late establishment and persistent adult expression of the Hoxa cluster genes. *Biol Reprod* 1997;**57:**1338-45.
44. Cheng W, Liu J, Yoshida H, Rosen D, Naora H. Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. *Nat Med* 2005;**11:**531-7.
45. Ozcan A, Liles N, Coffey D, Shen SS, Truong LD. PAX2 and PAX8 expression in primary and metastatic mullerian epithelial tumors: a comprehensive comparison. *Am J Surg Pathol* 2011;**35:**1837-47.
46. Visvader JE. Cells of origin in cancer. *Nature* 2011;**469:**314-22.
47. Jordan CT, Guzman ML, Noble M. Cancer stem cells. *N Engl J Med* 2006;**355:**1253-61.
48. Szotek PP, Chang HL, Brennand K, Fujino A, Pieretti-Vanmarcke R, et al. Normal ovarian surface epithelial label-retaining cells exhibit stem/progenitor cell characteristics. *Proc Natl Acad Sci U S A* 2008;**105:**12469-73.
49. Flesken-Nikitin A, Hwang CI, Cheng CY, Michurina TV, Enikolopov G, et al. Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche. *Nature* 2013;**495:**241-5.

50. Wang Y, Sacchetti A, van Dijk MR, van der Zee M, van der Horst PH, et al. Identification of quiescent, stem-like cells in the distal female reproductive tract. *PLoS One* 2012;**7:**e40691.
51. Roelofsen T, van Kempen LC, van der Laak JA, van Ham MA, Bulten J, et al. Concurrent endometrial intraepithelial carcinoma (EIC) and serous ovarian cancer: can EIC be seen as the precursor lesion? *Int J Gynecol Cancer* 2012;**22:**457-64.
52. Chan RW, Schwab KE, Gargett CE. Clonogenicity of human endometrial epithelial and stromal cells. *Biol Reprod* 2004;**70:**1738-50.
53. Dimitrov R, Timeva T, Kyurkchiev D, Stamenova M, Shterev A, et al. Characterization of clonogenic stromal cells isolated from human endometrium. *Reproduction* 2008;**135:**551-8.
54. Schwab KE, Chan RW, Gargett CE. Putative stem cell activity of human endometrial epithelial and stromal cells during the menstrual cycle. *Fertil Steril* 2005;**84 Suppl 2:**1124-30.
55. Schwab KE, Hutchinson P, Gargett CE. Identification of surface markers for prospective isolation of human endometrial stromal colony-forming cells. *Hum Reprod* 2008;**23:**934-43.
56. Gargett CE, Schwab KE, Zillwood RM, Nguyen HP, Wu D. Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium. *Biol Reprod* 2009;**80:**1136-45.
57. Cervello I, Gil-Sanchis C, Mas A, Delgado-Rosas F, Martinez-Conejero JA, et al. Human endometrial side population cells exhibit genotypic, phenotypic and functional features of somatic stem cells. *PLoS One* 2010;**5:**e10964.
58. Schuring AN, Schulte N, Kelsch R, Ropke A, Kiesel L, et al. Characterization of endometrial mesenchymal stem-like cells obtained by endometrial biopsy during routine diagnostics. *Fertil Steril* 2011;**95:**423-6.
59. Cervello I, Mas A, Gil-Sanchis C, Peris L, Faus A, et al. Reconstruction of endometrium from human endometrial side population cell lines. *PLoS One* 2011;**6:**e21221.
60. Chan RW, Gargett CE. Identification of label-retaining cells in mouse endometrium. *Stem Cells* 2006;**24:**1529-38.
61. Cervello I, Martinez-Conejero JA, Horcajadas JA, Pellicer A, Simon C. Identification, characterization and co-localization of label-retaining cell population in mouse endometrium with typical undifferentiated markers. *Hum Reprod* 2007;**22:**45-51.
62. Taylor HS. Endometrial cells derived from donor stem cells in bone marrow transplant recipients. *JAMA* 2004;**292:**81-5.
63. Du H, Taylor HS. Contribution of bone marrow-derived stem cells to endometrium and endometriosis. *Stem Cells* 2007;**25:**2082-6.
64. Vanderhyden BC, Shaw TJ, Ethier JF. Animal models of ovarian cancer. *Reprod Biol Endocrinol* 2003;**1:**67.
65. Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN, Nikitin AY. Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. *Cancer Res* 2003;**63:**3459-63.
66. Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, et al. Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. *Nat Med* 2005;**11:**63-70.
67. Pearce CL, Templeman C, Rossing MA, Lee A, Near AM, et al. Association between endometriosis and risk of histological subtypes of ovarian cancer: a pooled analysis of case-control studies. *Lancet Oncol* 2012;**13:**385-94.
68. Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med* 2010;**363:**1532-43.
69. Wu R, Hendrix-Lucas N, Kuick R, Zhai Y, Schwartz DR, et al. Mouse model of human ovarian endometrioid adenocarcinoma based on somatic defects in the Wnt/beta-catenin and PI3K/Pten signaling pathways. *Cancer Cell* 2007;**11:**321-33.
70. Clark-Knowles KV, Garson K, Jonkers J, Vanderhyden BC. Conditional inactivation of Brca1 in the mouse ovarian surface epithelium results in an increase in preneoplastic changes. *Exp Cell Res* 2007;**313:**133-45.
71. Kim KY, Park DW, Jeung EB, Choi KC. Conditional knockout of brca1/2 and p53 in mouse ovarian surface epithelium: do they play a role in ovarian carcinogenesis? *J Vet Sci* 2010;**11:**291-7.
72. Laviolette LA, Garson K, Macdonald EA, Senterman MK, Courville K, et al. 17beta-estradiol accelerates tumor onset and decreases survival in a transgenic mouse model of ovarian cancer. *Endocrinology* 2010;**151:**929-38.
73. Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, et al. Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. *Cancer Res* 2003;**63:**1389-97.

74. Liang S, Yang N, Pan Y, Deng S, Lin X, et al. Expression of activated PIK3CA in ovarian surface epithelium results in hyperplasia but not tumor formation. *PLoS One* 2009;**4:**e4295.
75. Fan HY, Liu Z, Paquet M, Wang J, Lydon JP, et al. Cell type-specific targeted mutations of Kras and Pten document proliferation arrest in granulosa cells versus oncogenic insult to ovarian surface epithelial cells. *Cancer Res* 2009;**69:**6463-72.
76. Mullany LK, Fan HY, Liu Z, White LD, Marshall A, et al. Molecular and functional characteristics of ovarian surface epithelial cells transformed by KrasG12D and loss of Pten in a mouse model in vivo. *Oncogene* 2011;**30:**3522-36.
77. Kim J, Coffey DM, Creighton CJ, Yu Z, Hawkins SM, et al. High-grade serous ovarian cancer arises from fallopian tube in a mouse model. *Proc Natl Acad Sci U S A* 2012;**109:**3921-6.
78. Tanwar PS, Kaneko-Tarui T, Lee HJ, Zhang L, Teixeira JM. PTEN loss and HOXA10 expression are associated with ovarian endometrioid adenocarcinoma differentiation and progression. *Carcinogenesis* 2013;**34:**893-901.
79. Gatcliffe TA, Monk BJ, Planutis K, Holcombe RF. Wnt signaling in ovarian tumorigenesis. *Int J Gynecol Cancer* 2008;**18:**954-62.
80. Van der Horst PH, Van der Zee M, Heijmans-Antonissen C, Jia Y, deMayo FJ, et al. Endometrioid ovarian cancer arising from the distal oviduct. *In submission* 2013.
81. Chen X, Aravindakshan J, Yang Y, Sairam MR. Early alterations in ovarian surface epithelial cells and induction of ovarian epithelial tumors triggered by loss of FSH receptor. *Neoplasia* 2007;**9:**521-31.
82. Chodankar R, Kwang S, Sangiorgi F, Hong H, Yen HY, et al. Cell-nonautonomous induction of ovarian and uterine serous cystadenomas in mice lacking a functional Brca1 in ovarian granulosa cells. *Curr Biol* 2005;**15:**561-5.
83. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, et al. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci U S A* 1998;**95:**13612-7.
84. Lauchlan SC. The secondary mullerian system revisited. *Int J Gynecol Pathol* 1994;**13:**73-9.
85. Bulun SE. Endometriosis. *New England Journal of Medicine* 2009;**360:**268-79.
86. D'Hooghe TM, Debrock S. Endometriosis, retrograde menstruation and peritoneal inflammation in women and in baboons. *Hum Reprod Update* 2002;**8:**84-8.
87. Kadar N, Krumerman M. Possible metaplastic origin of lymph node "metastases" in serous ovarian tumor of low malignant potential (borderline serous tumor). *Gynecol Oncol* 1995;**59:**394-7.
88. Rutgers JL, Scully RE. Cysts (cystadenomas) and tumors of the rete ovarii. *Int J Gynecol Pathol* 1988;**7:**330-42.
89. Quattropani SL. Serous cystadenoma formation in guinea pig ovaries. *J Submicrosc Cytol* 1981;**13:**337-45.
90. Auersperg N. Ovarian surface epithelium as a source of ovarian cancers: Unwarranted speculation or evidence-based hypothesis? *Gynecol Oncol* 2013.
91. Crum CP, Drapkin R, Miron A, Ince TA, Muto M, et al. The distal fallopian tube: a new model for pelvic serous carcinogenesis. *Curr Opin Obstet Gynecol* 2007;**19:**3-9.
92. Karst AM, Drapkin R. Ovarian cancer pathogenesis: a model in evolution. *J Oncol* 2010;**2010:**932371.
93. Mittal KR, Zeleniuch-Jacquotte A, Cooper JL, Demopoulos RI. Contralateral ovary in unilateral ovarian carcinoma: a search for preneoplastic lesions. *Int J Gynecol Pathol* 1993;**12:**59-63.
94. Pothuri B, Leitao MM, Levine DA, Viale A, Olshen AB, et al. Genetic analysis of the early natural history of epithelial ovarian carcinoma. *PLoS One* 2010;**5:**e10358.
95. Resta L, Russo S, Colucci GA, Prat J. Morphologic precursors of ovarian epithelial tumors. *Obstet Gynecol* 1993;**82:**181-6.
96. Bowen NJ, Walker LD, Matyunina LV, Logani S, Totten KA, et al. Gene expression profiling supports the hypothesis that human ovarian surface epithelia are multipotent and capable of serving as ovarian cancer initiating cells. *BMC Med Genomics* 2009;**2:**71.
97. Gamwell LF, Collins O, Vanderhyden BC. The mouse ovarian surface epithelium contains a population of LY6A (SCA-1) expressing progenitor cells that are regulated by ovulation-associated factors. *Biol Reprod* 2012;**87:**80.
98. Plaxe SC, Deligdisch L, Dottino PR, Cohen CJ. Ovarian intraepithelial neoplasia demonstrated in patients with stage I ovarian carcinoma. *Gynecol Oncol* 1990;**38:**367-72.
99. Kurman RJ, Shih Ie M. Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications. *Int J Gynecol Pathol* 2008;**27:**151-60.

# Chapter 5

## Endometrioid ovarian cancer arising from the distal oviduct

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# Chapter 6

## Malignant transformation of tubal precursors into serous ovarian cancer

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# Chapter 7

## General discussion

In this thesis, we describe our investigation on the mechanisms involved in the initiation and progression of Müllerian duct derived malignancies. The research was focused on the role of progesterone signaling in the progression of endometrial cancer (chapter 2, 3) and on the origin of epithelial ovarian cancer (chapter 4, 5 and 6). For this, 3 aims were described:

1. What is the effect of progesterone receptor signaling on the tumor specific immune response, Epithelial-Mesenchymal Transition (EMT) and recurrence in endometrial cancer?
2. What is the effect of activation of WNT/β-catenin signaling on Müllerian duct derived tissues?
3. Are Müllerian duct derived tissues the origin of epithelial ovarian cancer, can we initiate ovarian cancer from these tissues and can we identify and characterize tubal precursor lesions of serous ovarian carcinoma in controls, patients susceptible for and patients with serous ovarian cancer?

## Progesterone signaling stimulates infiltration of T-lymphocytes and inhibits epithelial-to-mesenchymal transition in endometrial cancer.

In general, patients with endometrial cancer have a good prognosis due to the fact that the disease is usually diagnosed at an early stage, in which it has not spread beyond the uterus[1]. However, if there is recurrent or metastatic disease, the situation is very different and progressive disease has a very poor prognosis accounting for 74.000 deaths worldwide each year[2]. Therefore, in order to improve therapy it is vital to understand the processes that inhibit and stimulate endometrial cancer progression. The research performed in chapter 3 aimed to investigate two mechanisms involved in metastatic spread of endometrial cancer: tumor infiltrating lymphocytes and progesterone induced inhibition of EMT. For this, primary endometrial cancer specimens from progressive and non-progressive endometrial cancer patients were assessed for the presence of CD4+ (helper), CD8+ (cytotoxic) and FOXP3+ (regulatory) T-lymphocytes and PR expression. As expected[3-5], patients with progressive (recurrent and/or metastatic) disease, showed a significant decrease in tumor infiltrating lymphocytes coinciding with loss of PR expression. Conformingly, gene expression analysis of frozen tumor samples of these patients, showed significant regulation of pathways involved in immunesurveillance, in addition to pathways involved in EMT and metastasis. Interestingly, inhibitors of WNT/β-catenin signaling, DKK1, DKK4 and WIF1, were down regulated in progressive disease, which was confirmed by quantitative RT-PCR analysis. These results were in line with our previous investigations, which showed that WNT/β-catenin signaling becomes activated at the same time as the progesterone receptor is lost[6].

In order to substantiate the finding that loss of progesterone signaling in progressive disease plays a role in diminished T-cell infiltration and induction of EMT, well differentiated Ishikawa endometrial cell lines stably transfected with PRA (IKPRA-1), PRB (IKPRB-1) and PRA+PRB (IKPRAB-36) were cultured in the presence or absence of progesterone (MPA) and subsequently used for immunohistochemistry, wound healing and modified Boyden chamber migration assay, and genome wide gene expression analysis. Culture of IKPRB-1 and IKPRAB-36, but not IKPRA-1, in

the presence of MPA resulted in inhibition of migration and down regulation of the mesenchymal marker vimentin at the invasive front of the wound. Furthermore, as in progressive disease, progesterone stimulated immunosuppression, but inhibited pathways and genes involved in EMT and metastasis: such as Integrin/ILK, EGF, PDGF, TGF-β and WNT/β-catenin-signaling. Interestingly, many of the differentially expressed signaling pathways in the Ishikawa cell lines, were also significantly altered in the patient samples.

In summary, we conclude that loss of progesterone signaling in progressive endometrial cancer causes a decrease in tumor infiltrating lymphocytes numbers and induces a transition from an epithelial to a more mesenchymal, more invasive phenotype in vivo, as well as in vitro.

## Activation of WNT/β-catenin signaling in Müllerian duct derived tissues causes endometrioid ovarian cancer.

As described in chapter 2, tight control of WNT/β-catenin signaling is crucial for the embryonic initiation and development of the Müllerian duct, cycle-depended proliferation and differentiation of the endometrium during reproductive life, and proper implantation and placenta formation during pregnancy. However, unbalanced WNT/β-catenin signaling is associated with endometriosis, endometrial hyperplasia and endometrial cancer. Due to its contribution in Müllerian duct development[7,8], many investigators studied the role of WNT/β-catenin signaling in ovarian carcinogenesis. As in endometrial cancer, WNT/β-catenin signaling was found to be an important factor in progression, metastasis, survival and chemoresistance of epithelial ovarian cancer[9-14]. Furthermore, several WNT-associated genes, *WNT5A, WNT7A* and *WNT9B*, were highly expressed in epithelial ovarian cancer[11-15] and endometrioid ovarian cancers frequently show gene mutations in *CTNNB1* and *APC*[16-21].

Knowing that WNT/β-catenin signaling plays an important role in endometrioid ovarian cancer and in view of the hypothesis that ovarian cancer may originate from Müllerian derived tissues, we studied mice in which WNT/β-catenin signaling was activated in Müllerian derived tissues (chapter 5). Here, *Pgr*-Cre induced mutation of *APC* resulted in the activation of WNT/β-catenin signaling in tissues derived from the Müllerian duct and granulosa cells, but not the OSE or ovarian stroma. In the oviducts of these mice, but not the uterus or OSE, precursor lesions were found that resembled human tubal intra-epithelial carcinoma (TIC). Over time and through a process of glandular transition, these precursor lesions developed into endometrial tubal tumors, which resembled human endometrioid tubal cancer. Interestingly, while no abnormalities were found in the OSE, starting from 10 weeks of age, simple endometrioid ovarian cysts were present. Over time, these cysts developed into large endometrioid ovarian tumors that resembled human endometrioid ovarian cancer. In addition, in 9,4% of mice, loco-regional spread to the uterine-ovarian ligament was observed.

These findings are in clear contrast with ovarian cancer models that appoint the OSE as a credible source of ovarian carcinoma. Interestingly, mouse models aiming to induce ovarian cancer from the OSE, either do not show epithelial ovarian cancer[22-25], recombine cells in both the oviduct

and OSE making discrimination between origins very difficult[26-28], or do not addresses possible Müllerian involvement[29-32]. Therefore, together with our recent finding of stem-like cells located in the distal oviduct[33], these findings strengthen the hypothesis that the Müllerian duct is the origin of ovarian cancer and the current mouse model can be a valuable tool for further research on ovarian cancer initiation, behavior and therapy.

## Malignant transition of tubal precursors into serous ovarian cancer.

To further substantiate the Müllerian origin of ovarian cancer, we studied the prevalence and characteristics of tubal precursor lesions of serous ovarian cancer (chapter 6). In this study, early benign (P53 signatures), intermediate (serous tubal intra-epithelial lesions, STILs) and clearly malignant (serous tubal intra-epithelial carcinomas, STICs) precursors of high grade serous ovarian cancer were identified with a hierarchy in prevalence from control, to patients at risk, to patients with a concurrent serous ovarian cancer. In the control group, P53 signatures were present in 6,7% of cases and in patients with a *BRCA* mutation this incidence increased to 26,7% for *BRCA1* and 46,7% for *BRCA2*. However, in none of these patients, lesions of malignant potential, STILs and STICs, were identified. Although P53 signature prevalence in *BRCA* gene mutation carriers is comparable with other studies, the absence of malignant lesions in this group was inconsistent[34-36]. Medeiros et al., 2006, identified STICs in 30% of tubal specimens collected during pBSO of *BRCA* gene mutation carriers. However, this high prevalence appears to be an exception as the prevalence of STICs in pBSO patients in many other studies usually varies between 1% and 6%[36-41].

Finally, serous ovarian carcinoma patients with or without a *BRCA* gene mutation were screened for tubal lesions. As expected, these patients showed a considerable increase in P53 signature prevalence and only here STILs, STICs and tubal adenocarcinomas were detected. P53 signatures were identified in 47% of cases and in addition to P53 signatures, STILs, STICs and tubal carcinomas were detected with a prevalence of 15,8%, 52,6% and 31,6% respectively. Furthermore, as indicated by several other studies[36, 42], tubal precursors were most commonly located in the fimbrial end of the fallopian tubes. Interestingly, in patients with a STIC, P53 signature prevalence was notably higher than in patients without a STIC. Further affirming the relationship between P53 signatures and STIC was the presence of P53 signatures and STILs aside STIC in a patient with concurrent serous ovarian carcinoma.

Upon further characterization of the identified STICs, a high resemblance of STIC to serous ovarian carcinoma was found on a morphological and molecular level. Using immunohistochemical analyses, STICs as well as concurrent ovarian cancer, showed enhanced WT1 and CA125 expression, decreased ERα and PRab expression and strong reduction of the mesenchymal marker vimentin. Furthermore, in STILs and STICs, membranous E-cadherin and β-catenin function was somewhat reduced, which indicates evidence of epithelial-to-mesenchymal transition.

In conclusion, our results support the hypothesis that serous ovarian cancer originates from lesions in the fallopian tube. Using a well-defined protocol (SEE-FIM) for total embedding of the

oviduct, benign, intermediate and malignant precursor tubal lesions of serous ovarian cancer were identified. Upon identification, immunohistochemical analysis confirmed the malignant and metastatic potential of STICs and further indicated its contributory relation as the origin of serous ovarian cancer.

## The Müllerian duct as origin of epithelial ovarian cancer.

Upon reviewing current literature and research described in this thesis, we appoint two possible mechanisms in which epithelial ovarian cancer arises based on the two pathway model of Kurman and Shih(2008)[43].

First, since type I ovarian tumors are typically ovarian confined and develop from borderline precursors, we hypothesize that these are derived from the oviduct or components of the secondary Müllerian system, such as ovarian endosalpingiosis or endometriosis, which over time acquire further genetical disturbances due to ovulation-induced distress or stimuli from the ovarian stroma.

Second, type II high grade serous ovarian carcinomas are mainly confined to the ovary and are characterized by mutation of *TP53*. Therefore we hypothesize that ovulation-induced mechanical, inflammatory and biochemical damage to the nearby distal fallopian tube epithelium results in areas mutated for *TP53* (P53 signatures). Upon further genetic damage and increased proliferation, P53 signatures develop into STILs, which, progress to become STICs. When transformed, malignant STIC cells can exfoliate and, in addition to other peritoneal sites, implant on or in the ovary. As a result, type II high grade serous ovarian cancer can develop.

In conclusion, not the OSE but the Müllerian duct should be appointed as the origin of epithelial ovarian cancer and research aiming to unravel the earliest carcinogenic changes in Müllerian derived tissues is key to facilitate early detection and targeted therapy for ovarian cancer.

## Future research into the Müllerian origin of ovarian cancer:

In order to further investigate the origin of ovarian cancer and to be able to detect and treat early lesions of epithelial ovarian cancer, a number of important research questions have to be answered.

1. Are the stem-like cells observed in the distal oviduct in mice truly stem cells and if so, for which tissues do they serve as stem cells?
2. Can these ductal stem cells, when mutated, serve as progenitor cells for epithelial ovarian cancer?
3. How can we translate our mice findings to facilitate improved management of ovarian carcinogenesis?

In order to proof stemness of the oviductal stem-like cells, lineage tracing needs to be developed. Lineage tracing however, is not as straightforward as one would hope. What is needed is a stem-like cell specific gene from which the promoter can be used to drive C-recombinase (Cre) expression. Stem-like cell specific Cre expression can then be used to drive reconstitution of a defective

reporter gene (YFP for example) essentially marking the stem-like cells and all cells derived from them[44-46].

Once a mouse model is available which specifically targets stem cells in the distal oviduct this model can now also be combined with conditionally mutated mice models. For example, combining oviductal stem cells specific Cre with *Apc*$^{lox}$ will most likely result in endometrioid ovarian cancer while combining it with *Brca1/2*$^{lox}$ and/or *P53*$^{lox}$ may induce serous ovarian cancer.

A significant challenge lies in translating animal data into human applications. For this the oviductal stem cells and early malignant precursors of ovarian cancer need to be analyzed in order to identify specific maker genes using genome wide expression analysis. From these ovarian cancer precursor specific genes, those which are upregulated and which encode proteins expressed at the cell surface will be selected. For these cell surface expressed proteins antibodies will be obtained and these antibodies will be labeled with a fluorophore. These labeled antibodies can be used for three applications. First these antibodies can be used to identify ovarian cancer precursors in vivo, second these antibodies can be used to isolate precursor cells (which can be used in transplantation experiments to proof carcinogenic properties of these cells) and, thirdly, these in vivo labeled cells can be removed using a sophisticated laser device. Furthermore, it is also possible that among these marker genes there will be biomarkers, which can be used to detect the presence of precursors using serum or other body fluids such as urine or menstrual blood.

Finally, upon review of the results from these future investigations, a large multicenter trial could be undertaken to assess the safety of salpingectomy without oophorectomy in patients predisposed for ovarian cancer (*BRCA1* and *BRCA2* carriers). If serous ovarian cancer only originates from the fallopian tube, salpingectomy should be sufficient to reduce the life-time ovarian cancer risk. Therefore, mastectomized patients should be randomly divided into two groups: 1: complete salpingo-oophorectomy at 40 years of age (standard protocol in the Erasmus MC) and with standard care; 2: salpingectomy alone at 30 years of age or after fulfilled child wish followed by oophorectomy after natural menopause. As a protective measure, in between salpingectomy and oophorectomy, patients should undergo intensive follow-up every 6 months by means of transvaginal sonogram, measurement of serum CA125 and possibly measurement of markers identified in the research described before. For patients predisposed for ovarian cancer, it is anticipated that, after a careful review of the success of the here suggested research program, prophylactic removal of the ovaries may no longer be necessary. This will immediately improve quality of life, since prophylactic removal of the ovary induces surgical menopause at young age, which is associated with increased cardiovascular risk, osteoporosis and declined psychological and sexual wellbeing[47-51].

# References


1. Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, et al. Endometrial cancer. *Lancet* 2005;**366:**491-505.
2. Ferlay J SH, Bray F, Forman D, Mathers C and Parkin DM. GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. [cited; Available from: http://globocan.iarc.fr, accessed on day/month/year.
3. de Jong RA, Leffers N, Boezen HM, ten Hoor KA, van der Zee AG, et al. Presence of tumor-infiltrating lymphocytes is an independent prognostic factor in type I and II endometrial cancer. *Gynecol Oncol* 2009;**114:**105-10.
4. Hanekamp EE, Gielen SC, Smid-Koopman E, Kuhne LC, de Ruiter PE, et al. Consequences of loss of progesterone receptor expression in development of invasive endometrial cancer. *Clin Cancer Res* 2003;**9:**4190-9.
5. Kondratiev S, Sabo E, Yakirevich E, Lavie O, Resnick MB. Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. *Clin Cancer Res* 2004;**10:**4450-6.
6. Wang Y, Hanifi-Moghaddam P, Hanekamp EE, Kloosterboer HJ, Franken P, et al. Progesterone inhibition of Wnt/beta-catenin signaling in normal endometrium and endometrial cancer. *Clin Cancer Res* 2009;**15:**5784-93.
7. van der Horst PH, Wang Y, van der Zee M, Burger CW, Blok LJ. Interaction between sex hormones and WNT/beta-catenin signal transduction in endometrial physiology and disease. *Mol Cell Endocrinol* 2012;**358:**176-84.
8. van der Horst PH, Burger CW, Blok LJ. Müllerian origin of ovarian cancer. *To be submitted* 2013.
9. Bitler BG, Nicodemus JP, Li H, Cai Q, Wu H, et al. Wnt5a suppresses epithelial ovarian cancer by promoting cellular senescence. *Cancer Res* 2011;**71:**6184-94.
10. Gatcliffe TA, Monk BJ, Planutis K, Holcombe RF. Wnt signaling in ovarian tumorigenesis. *Int J Gynecol Cancer* 2008;**18:**954-62.
11. Merritt MA, Parsons PG, Newton TR, Martyn AC, Webb PM, et al. Expression profiling identifies genes involved in neoplastic transformation of serous ovarian cancer. *BMC Cancer* 2009;**9:**378.
12. Peng C, Zhang X, Yu H, Wu D, Zheng J. Wnt5a as a predictor in poor clinical outcome of patients and a mediator in chemoresistance of ovarian cancer. *Int J Gynecol Cancer* 2011;**21:**280-8.
13. Varma RR, Hector SM, Clark K, Greco WR, Hawthorn L, et al. Gene expression profiling of a clonal isolate of oxaliplatin-resistant ovarian carcinoma cell line A2780/C10. *Oncol Rep* 2005;**14:**925-32.
14. Yoshioka S, King ML, Ran S, Okuda H, MacLean JA, 2nd, et al. WNT7A regulates tumor growth and progression in ovarian cancer through the WNT/beta-catenin pathway. *Mol Cancer Res* 2012;**10:**469-82.
15. Steg A, Wang W, Blanquicett C, Grunda JM, Eltoum IA, et al. Multiple gene expression analyses in paraffin-embedded tissues by TaqMan low-density array: Application to hedgehog and Wnt pathway analysis in ovarian endometrioid adenocarcinoma. *J Mol Diagn* 2006;**8:**76-83.
16. Gamallo C, Palacios J, Moreno G, Calvo de Mora J, Suarez A, et al. beta-catenin expression pattern in stage I and II ovarian carcinomas : relationship with beta-catenin gene mutations, clinicopathological features, and clinical outcome. *Am J Pathol* 1999;**155:**527-36.
17. Moreno-Bueno G, Gamallo C, Perez-Gallego L, de Mora JC, Suarez A, et al. beta-Catenin expression pattern, beta-catenin gene mutations, and microsatellite instability in endometrioid ovarian carcinomas and synchronous endometrial carcinomas. *Diagn Mol Pathol* 2001;**10:**116-22.
18. Palacios J, Gamallo C. Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. *Cancer Res* 1998;**58:**1344-7.
19. Saegusa M, Okayasu I. Frequent nuclear beta-catenin accumulation and associated mutations in endometrioid-type endometrial and ovarian carcinomas with squamous differentiation. *J Pathol* 2001;**194:**59-67.
20. Wright K, Wilson P, Morland S, Campbell I, Walsh M, et al. beta-catenin mutation and expression analysis in ovarian cancer: exon 3 mutations and nuclear translocation in 16% of endometrioid tumours. *Int J Cancer* 1999;**82:**625-9.
21. Wu R, Zhai Y, Fearon ER, Cho KR. Diverse mechanisms of beta-catenin deregulation in ovarian endometrioid adenocarcinomas. *Cancer Res* 2001;**61:**8247-55.

22. Clark-Knowles KV, Garson K, Jonkers J, Vanderhyden BC. Conditional inactivation of Brca1 in the mouse ovarian surface epithelium results in an increase in preneoplastic changes. *Exp Cell Res* 2007;**313:**133-45.
23. Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN, Nikitin AY. Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. *Cancer Res* 2003;**63:**3459-63.
24. Kim KY, Park DW, Jeung EB, Choi KC. Conditional knockout of brca1/2 and p53 in mouse ovarian surface epithelium: do they play a role in ovarian carcinogenesis? *J Vet Sci* 2010;**11:**291-7.
25. Liang S, Yang N, Pan Y, Deng S, Lin X, et al. Expression of activated PIK3CA in ovarian surface epithelium results in hyperplasia but not tumor formation. *PLoS One* 2009;**4:**e4295.
26. Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, et al. Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. *Cancer Res* 2003;**63:**1389-97.
27. Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, et al. Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. *Nat Med* 2005;**11:**63-70.
28. Tanwar PS, Kaneko-Tarui T, Lee HJ, Zhang L, Teixeira JM. PTEN loss and HOXA10 expression are associated with ovarian endometrioid adenocarcinoma differentiation and progression. *Carcinogenesis* 2013;**34:**893-901.
29. Fan HY, Liu Z, Paquet M, Wang J, Lydon JP, et al. Cell type-specific targeted mutations of Kras and Pten document proliferation arrest in granulosa cells versus oncogenic insult to ovarian surface epithelial cells. *Cancer Res* 2009;**69:**6463-72.
30. Laviolette LA, Garson K, Macdonald EA, Senterman MK, Courville K, et al. 17beta-estradiol accelerates tumor onset and decreases survival in a transgenic mouse model of ovarian cancer. *Endocrinology* 2010;**151:**929-38.
31. Mullany LK, Fan HY, Liu Z, White LD, Marshall A, et al. Molecular and functional characteristics of ovarian surface epithelial cells transformed by KrasG12D and loss of Pten in a mouse model in vivo. *Oncogene* 2011;**30:**3522-36.
32. Wu R, Hendrix-Lucas N, Kuick R, Zhai Y, Schwartz DR, et al. Mouse model of human ovarian endometrioid adenocarcinoma based on somatic defects in the Wnt/beta-catenin and PI3K/Pten signaling pathways. *Cancer Cell* 2007;**11:**321-33.
33. Wang Y, Sacchetti A, van Dijk MR, van der Zee M, van der Horst PH, et al. Identification of quiescent, stem-like cells in the distal female reproductive tract. *PLoS One* 2012;**7:**e40691.
34. Jarboe E, Folkins A, Nucci MR, Kindelberger D, Drapkin R, et al. Serous carcinogenesis in the fallopian tube: a descriptive classification. *Int J Gynecol Pathol* 2008;**27:**1-9.
35. Lee Y, Miron A, Drapkin R, Nucci MR, Medeiros F, et al. A candidate precursor to serous carcinoma that originates in the distal fallopian tube. *J Pathol* 2007;**211:**26-35.
36. Medeiros F, Muto MG, Lee Y, Elvin JA, Callahan MJ, et al. The tubal fimbria is a preferred site for early adenocarcinoma in women with familial ovarian cancer syndrome. *Am J Surg Pathol* 2006;**30:**230-6.
37. Callahan MJ, Crum CP, Medeiros F, Kindelberger DW, Elvin JA, et al. Primary fallopian tube malignancies in BRCA-positive women undergoing surgery for ovarian cancer risk reduction. *J Clin Oncol* 2007;**25:**3985-90.
38. Leeper K, Garcia R, Swisher E, Goff B, Greer B, et al. Pathologic findings in prophylactic oophorectomy specimens in high-risk women. *Gynecol Oncol* 2002;**87:**52-6.
39. Mingels MJ, Roelofsen T, van der Laak JA, de Hullu JA, van Ham MA, et al. Tubal epithelial lesions in salpingo-oophorectomy specimens of BRCA-mutation carriers and controls. *Gynecol Oncol* 2012;**127:**88-93.
40. Powell CB, Chen LM, McLennan J, Crawford B, Zaloudek C, et al. Risk-reducing salpingo-oophorectomy (RRSO) in BRCA mutation carriers: experience with a consecutive series of 111 patients using a standardized surgical-pathological protocol. *Int J Gynecol Cancer* 2011;**21:**846-51.
41. Reitsma W, de Bock GH, Oosterwijk JC, Bart J, Hollema H, et al. Support of the 'fallopian tube hypothesis' in a prospective series of risk-reducing salpingo-oophorectomy specimens. *Eur J Cancer* 2013;**49:**132-41.
42. Kindelberger DW, Lee Y, Miron A, Hirsch MS, Feltmate C, et al. Intraepithelial carcinoma of the fimbria and pelvic serous carcinoma: Evidence for a causal relationship. *Am J Surg Pathol* 2007;**31:**161-9.
43. Kurman RJ, Shih Ie M. Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications. *Int J Gynecol Pathol* 2008;**27:**151-60.
44. Schepers AG, Snippert HJ, Stange DE, van den Born M, van Es JH, et al. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science* 2012;**337:**730-5.

45. Alcolea MP, Jones PH. Tracking cells in their native habitat: lineage tracing in epithelial neoplasia. *Nat Rev Cancer* 2013;**13:**161-71.
46. Buczacki SJ, Zecchini HI, Nicholson AM, Russell R, Vermeulen L, et al. Intestinal label-retaining cells are secretory precursors expressing Lgr5. *Nature* 2013;**495:**65-9.
47. Castelo-Branco C, Palacios S, Combalia J, Ferrer M, Traveria G. Risk of hypoactive sexual desire disorder and associated factors in a cohort of oophorectomized women. *Climacteric* 2009;**12:**525-32.
48. Colditz GA, Willett WC, Stampfer MJ, Rosner B, Speizer FE, et al. Menopause and the risk of coronary heart disease in women. *N Engl J Med* 1987;**316:**1105-10.
49. Hreshchyshyn MM, Hopkins A, Zylstra S, Anbar M. Effects of natural menopause, hysterectomy, and oophorectomy on lumbar spine and femoral neck bone densities. *Obstet Gynecol* 1988;**72:**631-8.
50. Kelsey JL, Prill MM, Keegan TH, Quesenberry CP, Jr., Sidney S. Risk factors for pelvis fracture in older persons. *Am J Epidemiol* 2005;**162:**879-86.
51. Shifren JL. Androgen deficiency in the oophorectomized woman. *Fertil Steril* 2002;**77 Suppl 4:**S60-2.

# Chapter 8

**Summary**
**Samenvatting**

# Summary

The main goal of the work presented in this thesis is to unravel the mechanisms involved in the initiation and progression of Müllerian duct derived malignancies.

**Chapter 1** provides a general introduction of the female reproductive tract, endometrial and ovarian carcinoma and the aims of the study.

**Chapter 2** reviews the role of WNT/β-catenin signaling in the female reproductive tract, especially focusing on its interaction with sex hormones during embryonic development, pregnancy, endometriosis and endometrial cancer. It was concluded that tight control of WNT/β-catenin signaling is crucial for the embryonic initiation and development of the Müllerian duct, cycle-depended proliferation and differentiation of the endometrium during reproductive life, and proper implantation and placenta formation during pregnancy. However, if WNT/β-catenin signaling is not maintained in control, it may initiate endometriosis, endometrial hyperplasia and endometrial carcinoma.

The role of progesterone receptor signaling involved in important pathways in endometrial cancer progression was assessed in **Chapter 3**. In this study, it was observed that progression (recurrence and/or metastasis) of disease in endometrial cancer patients is characterized by loss of progesterone signaling, loss of tumor infiltrating T-lymphocytes and significant inhibition of pathways involved in immune surveillance and stimulation of pathways and genes involved in epithelial-to-mesenchymal transition and metastasis. In order to substantiate the role of progesterone signaling, Ishikawa endometrial cancer cell lines stably transfected with PRA(IKPRA-1), PRB(IKPRB-1) or PRA and PRB(IKPRAB-36) were subsequently cultured in presence/absence of progesterone (medroxyprogesterone acetate, MPA). Here, culture of IKPRB and IKPRAB in the presence of MPA resulted in inhibition of migration and downregulation of the mesenchymal marker vimentin. Furthermore, progesterone stimulated immunosuppression, but inhibited pathways and genes involved in EMT and metastasis. Based on these results it was concluded that loss of progesterone signaling in progressive endometrial cancer causes a decrease in tumor infiltrating lymphocyte numbers and induces a transition from an epithelial to a more mesenchymal, more invasive phenotype.

Epithelial ovarian cancer is the deadliest gynecological malignancy in Western countries, which is mainly caused by the fact that the origin of ovarian cancer and consequently its therapeutic approach, is still under debate. Therefore, **Chapter 4** extensively reviews the clinical and more basic research that has been performed to reveal the origin of ovarian cancer and unravel the process of early carcinogenesis. Here it was concluded, that not the ovarian surface epithelium (OSE), but the Müllerian duct should be appointed as the origin of epithelial ovarian cancer.

Knowing that in a high percentage of endometrioid ovarian cancers WNT/β-catenin signaling is activated, and in view of the hypothesis that ovarian cancer originates from the Müllerian duct, in **Chapter 5** we studied mice in which WNT/β-catenin signaling was conditionally activated in Müllerian duct derived tissues. These *Pgr*$^{cre/+}$;*Apc*$^{ex15lox/lox}$ mice developed tubal intraepithelial carcinomas (TICs), which, through a process of glandular transition, developed into endometrioid

tubal tumors. In the ovaries, mainly at young age, simple epithelial cysts were noted that developed further into endometrioid ovarian tumors, resembling human endometrioid ovarian cancer. Furthermore, loco-regional spread to the utero-ovarian ligament was shown. Since the OSE was not affected in these mice, it was concluded that endometrioid ovarian cancer develops from precursor lesions in the oviduct.

In order to further investigate the Müllerian origin of epithelial ovarian cancer, in **chapter 6** we determined the prevalence and characteristics of tubal precursor lesions in patients with serous ovarian cancer, with susceptibility for serous ovarian cancer as well as healthy controls. In this study a hierarchy in prevalence of lesions from controls, to patients with an increased risk, to patients with serous ovarian cancer was identified. However, while "benign" P53 signatures were found in all groups, precursors considered of malignant potential, STILs and STICs, were only found in patients with serous ovarian cancer. Furthermore, STICs showed similar characteristics as concurrent ovarian carcinoma and some evidence of epithelial-to-mesenchymal transition in STICs was found, making metastatic spread of malignant tubal cells to the ovary plausible. Therefore, it was concluded that serous ovarian cancer originates from precursor lesions in the oviduct.

**Chapter 7 and 8** provide a summary of the results of the studies in this thesis and a general discussion. Furthermore, directions for future research and possible clinical implications are assessed.

# Samenvatting

Het doel van het onderzoek beschreven in dit proefschrift is het ontrafelen van mechanismen die betrokken zijn bij het ontstaan en bij de progressie van maligniteiten van Müllerse gang afgeleide weefsels.

In **hoofdstuk 1** wordt een algemene inleiding over het vrouwelijke voortplantingssysteem, endometrium- en ovariumcarcinoom gegeven. Daarnaast beschrijft dit hoofdstuk de doelstellingen behorende bij dit proefschrift.

**Hoofdstuk 2** beschrijft de rol van WNT/β-catenine signalering in het vrouwelijke voortplantingssysteem en richt zich in het bijzonder op de interactie tussen WNT/β-catenine signalering en de werking van de vrouwelijke geslachtshormonen oestradiol en progesteron tijdens embryonale ontwikkeling, normale fysiologie, zwangerschap, endometriose en endometriumkanker. Geconcludeerd werd dat nauwkeurige regulatie van WNT/β-catenine signalering cruciaal is voor de initiatie en ontwikkeling van de Müllerse gang tijdens de embryogenese, de menstruele cyclus, de innesteling van het embryo en de vorming van de placenta tijdens de zwangerschap. Wanneer WNT/β-catenine signalering niet goed wordt gereguleerd kunnen endometriose, endometriumhyperplasie en endometriumkanker ontstaan.

In **hoofdstuk 3** wordt de rol van progesteron en de progesteronreceptoren (PR) in relatie tot de progressie van endometriumcarcinoom onderzocht. In deze studie werd waargenomen dat progressie (recidivering en/of metastasering) van endometriumcarcinoom wordt gekenmerkt door het verlies van progesteron werking, verlies van tumor-infiltrerende T-lymfocyten, een significante remming van signaleringssystemen betrokken bij de immuunrespons en stimulering van signaleringssystemen en genen betrokken bij epitheliale naar mesenchymale transitie (EMT) en metastase. Om de rol van progesteron verder te onderzoeken werden Ishikawa endometriumcarcinoom cellijnen stabiel getransfecteerd met de A-vorm van de progesteronreceptor (IKPRA-1), de B-vorm van de progesteronreceptor (IKPRB-1) of de A- en B-vorm van de progesteronreceptor (IKPRAB-36), en vervolgens gekweekt in aan- of afwezigheid van progesteron (medroxyprogesteronacetaat, MPA). Het kweken van IKPRB en IKPRAB in aanwezigheid van MPA resulteerde in remming van celmigratie en verminderde expressie van de mesenchymale marker vimentine. Bovendien stimuleerde progesteron de immuunrespons en remde signaleringssystemen en genen betrokken bij EMT en metastase. Aan de hand van deze resultaten werd geconcludeerd dat verlies van progesteron werking in progressief endometriumcarcinoom een verlaging van de lokale immuunrespons en een overgang van een epitheliaal naar een mesenchymaal, meer invasief fenotype, initieert.

Epitheliaal ovariumcarcinoom is de dodelijkste gynaecologische maligniteit in westerse landen. Deze hoge mortaliteit wordt voornamelijk veroorzaakt door het feit dat de oorsprong van ovariumcarcinoom nog ter discussie staat, waardoor vroege diagnose en gerichte therapeutische benadering zeer moeilijk zijn. In **hoofdstuk 4** beschrijven we uitvoerig het klinisch en fundamenteel onderzoek dat is uitgevoerd naar het ontstaan van ovariumcarcinoom. Geconcludeerd werd dat

niet het ovariële oppervlakte-epitheel maar weefsels afkomstig vanuit de Müllerse gang moeten worden aangewezen als de oorsprong van epitheliaal ovariumcarcinoom.

Omdat in een hoog percentage van de endometrioide ovariumcarcinomen het WNT/β-catenine signaleringssysteem is geactiveerd en gezien de hypothese dat ovariumcarcinoom afkomstig zou kunnen zijn vanuit weefsels van de Müllerse gang, hebben we in **hoofdstuk 5** muizen bestudeerd waarin WNT/β-catenine signalering is geactiveerd in weefsels afkomstig van de Müllerse gang. Deze *Pgr*$^{Cre/+}$;*Apc*$^{ex15lox/lox}$ muizen ontwikkelden tubaire intra-epitheliale carcinomen (TIC) welke, door middel van een proces van glandulaire transitie, zich ontwikkelden tot endometrioïde tubaire tumoren. Daarnaast vonden wij in de ovaria van deze muizen, eenvoudige endometrioïde cysten die zich verder ontwikkelden tot endometrioïde ovariële tumoren die grote gelijkenis vertonen met humaan endometrioïd ovariumcarcinoom. Bovendien, werd locoregionale verspreiding van de endometrioïde tumoren in het utero-ovariële ligament aangetoond. Aangezien het ovariële oppervlakte-epitheel in deze muizen niet gemuteerd wordt, concluderen wij aan de hand van deze resultaten dat endometrioïd ovarium carcinoom ontwikkelt vanuit precursor laesies in de tuba.

Om verder te onderzoeken of weefsels van de Müllerse gang de oorsprong zijn van epitheliaal ovarium carcinoom, hebben we in **hoofdstuk 6** de prevalentie en kenmerken van tubaire precursor laesies onderzocht in patiënten met sereus ovariumcarcinoom, patiënten met een verhoogd erfelijk risico op sereus ovariumcarcinoom en gezonde controles. In deze studie werd een oplopende prevalentie van laesies gevonden van controles, naar patiënten met een verhoogd erfelijk risico, naar patiënten met sereus ovariumcarcinoom. Verder bleek dat (pre)maligne STILs en STICs alleen werden gevonden bij patiënten met een sereus ovariumcarcinoom, terwijl de "goedaardige" P53 signatures aanwezig waren in alle groepen. Bovendien vertoonde de gevonden TICs dezelfde moleculaire kenmerken als het bijbehorende ovariumcarcinoom. Daarnaast vonden we aanwijzingen van epitheliale-naar-mesenchymale transitie in de TICS, wat de metastatische verspreiding van kwaadaardige cellen van de tuba naar het ovarium plausibel maakt. Derhalve werd geconcludeerd dat sereus ovariumcarcinoom afkomstig is van precursor laesies in de tuba.

**Hoofdstuk 7 en 8** vormen de samenvatting van de resultaten van de studies beschreven in dit proefschrift en een algemene discussie. Verder worden aanwijzingen voor toekomstig onderzoek en mogelijke klinische implicaties gegeven.

# Appendices

# List of abbreviations

| | |
|---|---|
| Ad-Cre | Adenoviral C-recombinase |
| ALDH1 | Aldehyde dehydrogenase |
| AMH | Anti-Müllerian hormone |
| APC | Adenomatosis polyposis coli |
| ARID1a | AT rich interactive domain 1A |
| BMP2 (4,…) | Bone morphogenetic protein 2 |
| BRCA1 | Breast cancer 1, early onset |
| BRCA2 | Breast cancer 2, early onset |
| BSA | Bovine serum albumin |
| CA125 | Cancer antigen 125 |
| CCL21 | Chemokine (C-C motif) ligand 21 |
| CCR | C-C motif receptor |
| CD4 (8,…) | Cluster of differentiation 4 |
| CICs | Cortical inclusion cysts |
| CK1 | Casein kinase 1 |
| Ck6b | Cytokeratin-6B (mouse) |
| CRE | C-recombinase |
| CRUMBS3 | Crumbs protein homolog 3 |
| CTNNB1 | Catenin (cadherin-associated protein), beta 1 |
| CXCL9 (10,…) | Chemokine (C-X-C motif) ligand 9 |
| CXCR | C-X-C motif receptor |
| DAX1 | Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene |
| DKK1 | Dickkopf WNT signaling pathway inhibitor 1 |
| E12.5 | Embyonic day 12,5 |
| EGF | Epidermal growth factor |
| EIC | Endometrial intra-epithelial carcinoma |
| EMT | Epithelial-to-mesenchymal transition |
| ER | Estrogen receptor |
| ERBB-2 | V-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 |
| FACsorting | Fluorescence-activated cell sorting |
| FCS | Fetal calf serum |
| FGF | Fibroblast growth factor |
| FOXL2 | Forkhead box L2 |
| FOXO1 | Forkhead box O1 |
| FOXP3 | Forkhead box P3 |
| FSH | Follicle-stimulating hormone |
| FSHR | follicle-stimulating hormone receptor |

| | |
|---|---|
| FZD | Frizzled receptor |
| GFP | Green fluorescent protein |
| GnRH | Gonadotropin-releasing hormone |
| GSK3β | Glycogen synthase kinase 3 beta |
| HCG | Human chorionic gonadotropin |
| HE4 | Human epididymis protein 4 |
| HMGA2 | High mobility group AT-hook 2 |
| HNPCC | Hereditary nonpolyposis colorectal cancer |
| HOXA9 (10,…) | Homeobox A9 |
| H-Y | H-Y antigen |
| IGF | Insulin-like growth factor |
| IHC | Immunohistochemistry |
| IL2 (8,…) | Interleukin 2 |
| ILK | Integrin-linked kinase |
| INF-γ | Interferon-gamma |
| KLF8 | Kruppel-like factor 8 |
| KRAS | Kirsten rat sarcoma viral oncogene homolog |
| L1CAM | L1 cell adhesion molecule |
| LEF | Lymphoid enhancer-binding factor |
| LGR5 | Leucine-rich repeat containing G protein-coupled receptor 5 |
| LH | Luteinizing hormone |
| LIM1 | LIM homeobox 1 |
| LRCs | Label-retaining cells |
| MAPK | Mitogen-activated protein kinase |
| MLH1 | MutL homolog 1 |
| MMP2 (7,..) | Matrix metallopeptidase 2 |
| MPA | Medroxyprogesterone acetate |
| MSH2 (6,…) | MutS homolog 2 |
| MUC16 | Mucin 16 |
| OCT4 | Octamer-binding transcription factor 4 |
| OSE | Ovarian surface epithelium |
| (T)P53 (63,..) | Tumor protein p53 |
| PAEP | Progestagen-associated endometrial protein |
| PAX2 (8,…) | Paired box gene 2 |
| pBSO | Prophylactic bilateral salpingo-oophorectomy |
| PCOS | Polycystic ovary syndrome |
| PDGF | Platelet-derived growth factor |
| PEG10 | Paternally expressed 10 |
| PIK3CA | phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha |

| | |
|---|---|
| POU5F1 | POU domain, class 5, transcription factor 1 |
| PR | Progesterone receptor |
| PTEN | Phosphatase and tensin homolog |
| Rb1 | Retinoblastoma 1 (mouse) |
| RT | Room temperature |
| RT-PCR | Real-time polymerase chain reaction |
| SAM | Statistical analysis of microarray |
| SFRP4 | secreted frizzled-related protein 4 |
| SLUG | SLUG zinc-finger protein |
| SNAIL1 | Snail family zinc finger 1 |
| SRY | Sex-determining region Y |
| STICs | Serous tubal intra-epithelial carcinomas |
| STILs | Serous tubal intra-epithelial lesions |
| SV40 | Simian virus 40 |
| TCF | T-cell factor |
| TDF | Testis determining factor |
| TGF-β | transforming growth factor beta |
| TICs | Tubal intra-epithelial carcinoma |
| TILs | Tumor-infiltrating T-lymphocytes |
| TVU | Transvaginal ultrasonography |
| TWIST1 (2,…) | Twist basic helix-loop-helix transcription factor 1 |
| VEGF | Vascular endothelial growth factor |
| WIF1 | Wnt inhibitory factor 1 |
| WNT1 (1A,…) | Wingless-type MMTV integration site family, member 1 |
| WT1 | Wilms tumor 1 |
| γH2AX | gamma-H2A histone family, member X |
| ZEB1 (2,…) | Zinc finger E-box-binding homeobox 1 |
| ZFY | Zinc finger Y-chromosomal protein |

# PhD Portfolio


## Summary

### Summary of PhD training and teaching activities

| | |
|---|---|
| Name PhD student: **P.H. van der Horst** | PhD period: **2009-2013** |
| Erasmus MC Department: **Obstetrics and Gynaecology** | Promotor: **Prof. dr. C.W. Burger** |
| Research School: **Molecular medicine** | Copromotor: **Dr. ir. L.J. Blok** |

**1. PhD training**

| | Year | Workload (ECTS) |
|---|---|---|
| **General academic skills** | | |
| - Laboratory animal science (Art. 9 course) (6-9-2010 – 24-9-2010) Rotterdam | 2010 (second) | 4.50 |
| - Course on presentation skills (5-4, 26-4, 10-5-12) Rotterdam | 2012 (third) | 1.00 |
| **Research skills** | | |
| - Statistics (Basic Introduction Course on SPSS 10 & 11-06-2010 | 2010 (first) | 0.60 |
| - Molecular Diagnostics IV (28-5-2009 - 29-5-2009) Rotterdam | 2009 (first) | 1.00 |
| - Biomedical Research Techniques (12-10-09 - 16-10-09) Rotterdam | 2009 (first) | 1.60 |
| - Basic Data Analysis on Gene Expression Arrays (26-10-09 - 27-10-09) Rotterdam | 2009 (first) | 1.20 |
| - Radiation safety course 5A and 5B (2012) Rotterdam | 2012 (third) | 3.00 |
| - Basic course on using 'R' for data manipulation and statistical analyses | 2012 (fourth) | 1.40 |
| **In-depth courses (e.g. Research school, Medical Training)** | | |
| - Basic and Translational Oncology (09-11-2009 - 13-11-2009) Rotterdam | 2009 (first) | 1.80 |
| - Research Management (15-06-2010 & 29-06-2010) Rotterdam | 2010 (first) | 1.00 |
| - Photoshop and Illustrator CS5 course (29-3-11 - 30-3-11) Rotterdam | 2011 (second) | 0.30 |
| - InDesign CS5 course (13-04-11) Rotterdam | 2011 (second) | 0.20 |
| - Finance for non-financials (01-08-2011 – 05-08-2011), Nyenrode Business University Breukelen | 2011 (second) | 2.00 |

| | | |
|---|---|---|
| **Presentations** | | |
| - Presentation at the Leuven University Hospital (08-07-2009) | 2009 (first) | 0.50 |
| - Presentation at the SGGO meeting Rotterdam (29-07-2009) | 2009 (first) | 0.50 |
| - Presentation at the SGGO meeting Rotterdam (25-01-2010) | 2010 (first) | 0.50 |
| - Presentation at the Juriy Wladimiroff Symposium (12-03-2010) | 2010 (first) | 0.50 |
| - Presentation at the JNI scientific meeting (14-06-2010) | 2010 (first) | 0.50 |
| - Presentation at the Wetenschapslunch Cluster 12 (28-10-2010) | 2010 (second) | 0.50 |
| - Presentation at the Leuven University Hospital (29-10-2010) | 2010 (second) | 0.50 |
| - Presentation at the Gynaecongres (11-11-2010) | 2010 (second) | 0.50 |
| - Presentation at the JNI scientific meeting (06-06-2011) | 2011 (second) | 0.50 |
| - Presentation at the SEOHS Amsterdam (18-11-2011) | 2011 (third) | 0.50 |
| - Presentation at the JNI scientific meeting (12-12-2011) | 2011 (third) | 0.50 |
| - Presentation at the JNI scientific meeting (24-09-2012) | 2012 (fourth) | 0.50 |
| - Presentation at the Gynaecongres (15-11-2012) | 2012 (fourth) | 0.50 |
| - Presentation at the Science meeting cluster 15 (06-02-2013) | 2013 (fourth) | 0.50 |
| - Presentation at the MolMed Day (13-02-2013) | 2013 (fourth) | 0.50 |
| - Presentation at the Juriy Wladimiroff Symposium (15-03-2013) | 2013 (fourth) | 0.50 |
| - Presidents Elect Young Investigator Session, SGI, USA, 20-03-2013 | 2013 (fourth) | 0.50 |
| **International conferences** | | |
| - Ovarian Cancer Screening, London (UK) (29-11 – 30-11-2011) | 2011 (third) | 0.50 |
| - 2nd ESGO/ENTRIGO Translational Research Workshop, London (UK), (16-11-2012) | 2012 (fourth) | 0.25 |
| - SGI Annual Scientific Meeting, Orlando, Florida (USA) (20 – 23-03-2013) | 2013 (fourth) | 0.75 |
| **Seminars and workshops** | | |
| - 14th Molecular Medicine Day Rotterdam (04-03-2010) | 2010 (first) | 0.25 |
| - PhD day 2010 (20-05-2010) | 2010 (first) | 0.25 |
| - PhD day 2011 (27-05-2011) | 2011 (second) | 0.25 |
| - 16th Molecular Medicine Day Rotterdam (29-02-2012) | 2012 (third) | 0.25 |
| **Other** | | |
| - Organisation of the SEOHS symposium 2010 (19-11-2010) | 2010 (1st & 2nd) | 2.00 |

| 2. Teaching activities | | |
|---|---|---|
| | **Year** | **Workload (Hours/ ECTS)** |
| **Supervising practicals and excursions** | | |
| - Designing and supervising the Junior Science Program for Gynaecological Oncology, 2 high school students (16-11-09 - 20-11-09) Rotterdam | 2009 (first) | 1.00 |
| - Designing and supervising the Junior Science Program for Gynaecological Oncology, 2 high school students (21-06-10 - 25-06-10) Rotterdam | 2010 (first) | 1.00 |
| - Designing and supervising the Junior Science Program for Gynaecological Oncology / Pathology, 2 high school students (10-10-11 - 14-10-10) Rotterdam | 2011 (third) | 1.00 |
| **Supervising Master's theses** | | |
| - Substitute supervisor for a fourth year medical student elective research program (4 weeks, Matthijs van Dijk) | 2009 (first) | 0.50 |
| - Designing and supervising a master's thesis medical student elective research program (21 weeks, Nov - Jun, Ms. Sadé Daal) | 2011 (third) | 3.00 |
| - Designing and supervising a master's thesis medical student elective research program (21 weeks, Jul - Dec, Ms. Renske Wijnhoven) | 2012 (fourth) | 3.00 |
| - Designing and supervising a master's thesis medical student elective research program (21 weeks, Oct - Mar, Ms. Marthe Mouthaan) | 2012 (fourth) | 3.00 |
| - Designing and supervising a master's thesis medical student elective research program (17 weeks, Mar - Jul, Ms. Margot Cloostermans) | 2013 (fourth) | 3.00 |

# Publications and awards:

## Publications:

*Interaction between sexhormones and Wnt/β-catenin signal transduction in endometrial physiology and disease.*
Paul H. van der Horst, Yongyi Wang, Marten van der Zee, Curt W. Burger and Leen J. Blok
*Mol Cell Endocrinol* 2012;**358:**176-84.

*Progesterone inhibits epithelial-to-mesenchymal transition in endometrial cancer.*
Paul H. van der Horst, Yongyi Wang, Ingrid Vandenput, Liesbeth C. Kuhne, Patricia C. Ewing, Wilfred F.J. Van IJcken, Marten van der Zee, Frederic Amant, Curt W. Burger and Leen J. Blok
*PLoS One* 2012;**7(1)**: e30840

*Identification of quiescent stem- like cells in the distal female reproductive tract.*
Yongyi Wang, Andrea Sacchetti, Matthijs R. van Dijk, Marten van der Zee, Paul H. van der Horst, Rosalie Joosten, Curt W. Burger, J. Anton Grootegoed, Leen J. Blok and Ricardo Fodde
*PLoS One* 2012;**7:**e40691.

*Endometrioid ovarian cancer arising from the distal oviduct.*
Paul H. van der Horst, Marten van der Zee, Claudia Heijmans-Antonissen, Yundan Jia, Francesco J. DeMayo, John P. Lydon, Carlolien H.M. van Deurzen, Patricia C. Ewing, Curt W. Burger and Leen J. Blok.
*Submitted for publication*

*Malignant transition of tubal precursors into serous ovarian cancer.*
Paul H. van der Horst, Renske K.E. Wijnhoven, Sadé Daal, Marthe H. Mouthaan, Claudia Heijmans-Antonissen, Ronald van der Knaap, Ramon G.V. Smolders, Diederick de Jong, Jurgen M. Piek, Patricia C. Ewing, Curt W. Burger and Leen J. Blok.
*Submitted for publication*

*Müllerian origin of ovarian cancer.*
Paul H. van der Horst, Curt W. Burger and Leen J. Blok.
*In preparation*

*A rat model of anastomotic leakage created by insufficient sutures after colectomy.*
Zhouqiao Wu, G. Simone A. Boersma, King Lam, Paul H. van der Horst, Gert-Jan J. Kleinrensink, Johannes Jeekel, Johan F. Lange.
*Submitted for publication*

*Reinforcement of anastomosis by tissue adhesive in a contaminated environment.*
Zhouqiao Wu, Konstantinos A. Vakalopoulos, G. Simone A. Boersma, F. Daams, King Lam, Leen J. Blok, Paul H. van der Horst, Gert-Jan J. Kleinrensink, Johannes Jeekel, Johan F. Lange.
*Submitted for publication*

*An in vivo overview of the adhesive strength and healing effects of commercially available tissue adhesives.*
K.A. Vakalopoulos, Z. Wu, L. Kroese, P.H. van der Horst, L.J Blok, J. Jeekel, J.F. Lange.
*In preparation*

*Quality and quantity of memories in patients undergoing awake brain tumour resection.*
M. Klimek, P.H van der Horst, C. Müller, R.J. Stolker.
*In preparation*

## Awards:

Beste Jonge Onderzoeker tijdens het Gynaecongres van de Nederlandse Vereniging voor Obstetrie en Gynaecologie (NVOG) op 15 november 2012, Congrescentrum Papendal, Arnhem.

Giorgio Pardi Foundation Plenary Award for outstanding research by a junior investigator 2013, Giorgio Pardi Foundation, Milaan, Italië (uitgereikt tijdens de 2013 Annual World Meeting, Society of Gynecological Investigation (SGI), Orlando, Florida, USA).

Prof.dr. Juriy Wladimiroff Onderzoeksprijs 2013, Rotterdamse Gynaecologen Opleidingscluster (RGOC), Erasmus Universitair Medisch Centrum Rotterdam.

# About the author

Paul Henryk van der Horst was born in Rotterdam on April 21st, 1987. During secondary school ("atheneum"), he studied jazz drums from 2001 until 2004 at the Young Talent School of the Rotterdam Conservatory. After his graduation from secondary school in 2005, he enrolled in medical school at the Erasmus University Medical Center in Rotterdam and from that moment on he participated in scientific research. From 2005 until 2006 he performed an assay on patient experiences and awareness during the Awake Craniotomy at the department of Anesthesiology (supervision by Dr. M. Klimek). During this period he also worked on a trail for Near-Infrared Cerebral Oximetry during aortal aneurysm repair intervention procedures at the departments of Anesthesiology and Surgery (supervision by Dr. F. Grüne). From 2006 until 2008 he participated in a study on the prognostic value of homocysteine testing before and after methionine loading in predicting long-term mortality and major adverse cardiac events (Dept. of Surgery, supervision by Dr. M. Dunkelgrun). Since 2008, he is involved in the research conducted at the department of Obstetrics and Gynaecology. In 2009, he performed his 21-weeks elective research period on the immunological mechanisms of endometrial cancer and progesterone receptor gene expression profiling (supervision by Dr.ir. L.J. Blok and Dr. Y. Wang). After his doctoral graduation in August 2009, Paul started working as a fulltime PhD-student in this department (supervision by Prof. dr. C.W. Burger and Dr.ir. L.J. Blok). During this period he investigated the mechanisms involved in the initiation and progression of Müllerian derived malignancies with a special focus on the progression and recurrence of endometrial cancer and the origin, development and progression of epithelial ovarian cancer. During his PhD research, Paul was rewarded with a number of (inter) national awards and successfully published his results in international scientific journals.

# Dankwoord

Nu het proefschrift af is, rest mij als laatste het dankwoord. Misschien is dit nog wel het moeilijkste onderdeel van het boekje, want hoe bedank je iedereen die de afgelopen jaren heeft bijgedragen aan het tot stand komen van dit proefschrift en alles er omheen? Ik ga het toch proberen en als ik iemand vergeten ben, ook jij bedankt!

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Dan mijn paranimfen, mijn beste vrienden. Hidde, vriend van het eerste uur. We hebben alles samen meegemaakt, van absolute hoogtepunten tot de allergrootste dieptepunten. Ik weet zeker dat jij er komt en ik hoop dat onze vriendschap nog zeer lang mag duren. Konstantinos, we leerden elkaar kennen tijdens de Art. 9 cursus en het klikte meteen. Dank voor al je hulp, je vriendschap

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