Signaling pathways and stem cells in uterus and fallopian tubes

Yongyi Wang

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SIGNALING PATHWAYS AND STEM CELLS IN UTERUS AND FALLOPIAN TUBES

Signaaltransductie en stamcellen in uterus en oviduct

Thesis

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Yongyi Wang

Born in Dalian, China

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Promotors:	Prof. dr. J. A. Grootegoed
	Prof. dr. R. Fodde
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CONTENTS

Chapter 1	General introduction	7
Chapter 2	Loss of APC function in mesenchymal cells surrounding the Müllerian duct leads to myometrial defects in adult mice	29
Chapter 3	Progesterone inhibition of Wnt/β-catenin signaling in normal endometrium and endometrial cancer	47
Chapter 4	Identification of quiescent, stem-like cells in the distal female reproductive tract	77
Chapter 5	Discussion	101
Chapter 6	Summary Samenvatting 总结	117
	PhD portfolio	124
	Acknowledgements 感谢	126
	List of publications	129
	List of abbreviations	131
	About the author	

1

GENERAL INTRODUCTION

Adapted from two reviews:

Wang Y, van der Zee M, Fodde R, et al. Wnt/Beta-catenin and sex hormone signaling in endometrial homeostasis and cancer. *Oncotarget*. 2010;1:674-684.

van der Horst PH, Wang Y, van der Zee M, et al. Interaction between sexhormones and WNT/beta-catenin signal transduction in endometrial physiology and disease. *Mol Cell Endocrinol*. 2011 in press

1.1 DEVELOPMENT OF THE FEMALE REPRODUCTIVE TRACT

Wolffian (mesonephric) and Müllerian (paramesonephric) ducts are the developmental precursors of a major part of the male and female genital tracts, exclusive of the external genitalia and the gonads. During early mouse embryogenesis, Wolffian ducts are formed at embryonic day 9 (E9) from the intermediate mesoderm, and Müllerian ducts start to form around E11.5 from the coelomic epithelium[1-2] (Fig. 1). This occurs in embryos of both sexes. In female mouse embryos, in the coelomic epithelium, Lim1 expressing cells are induced to invaginate by Wnt4, which is expressed from the nearby Wolffian duct or from surrounding coelomic epithelial cells[3] (Fig. 1A and B). The transcription factor Lim1 is an essential regulator in development, and Lim1 knockout mice lack anterior head formation[4]. Furthermore, Lim1 knockout female mice lack a uterus and oviducts. Subsequently, invaginating cells start to proliferate and the structure elongates along the Wolffian duct. This elongation of the Müllerian ducts is mediated by Wnt9b expressing epithelial cells from the Wolffian duct[5] (Fig. 1C). Either in the absence of Wolffian ducts or in the absence of Wnt9b, the Müllerian ducts do not develop further. Outgrowth of the Müllerian duct is accomplished by proliferation of a group of coelomic epithelial cells resembling mesoepithelial cells at the distal tip[6-7]. At the end of elongation, both Müllerian ducts will fuse to form the uterovaginal tube, which joins the urogenital sinus thus starting to shape the upper part of the vagina.

Between E11.5 and E13.5, both Wolffian and Müllerian ducts preexist in the same embryo regardless of its genetic sex (Fig. 1D). After E13.5, however, the destiny of Wolffian and Müllerian ducts becomes determined by hormones secreted from the gonads[8-9]. In male sexual development, anti-Müllerian hormone (AMH) secreted by Sertoli cells in the testes binds to the AMH receptor (AMHR) on mesenchymal cells surrounding the Müllerian ducts[10], which results in apoptosis of the Müllerian ducts, while Wolffian ducts are stabilized and further develop (to become epididymis, vas deferens, seminal vesicle) under the influence of testosterone (Fig. 1F). In female sexual development, when there is no production of AMH by the fetal gonads, Müllerian ducts develop into the female reproductive tract. At the same time Wolffian ducts regress due to lack of testosterone[11] (Fig. 1E). Once initiated, correct patterning of the Müllerian ducts into upper part of the vagina, and the cervix, uterus and oviducts partly depends on epithelial cells of the oviduct and uterus expressing Wnt7a, and mesenchymal cells of the uterus, cervix and vagina expressing Wnt5a[12-13]. From the above, it is evident that there is a strong relationship between Wnt signaling and Müllerian duct development.

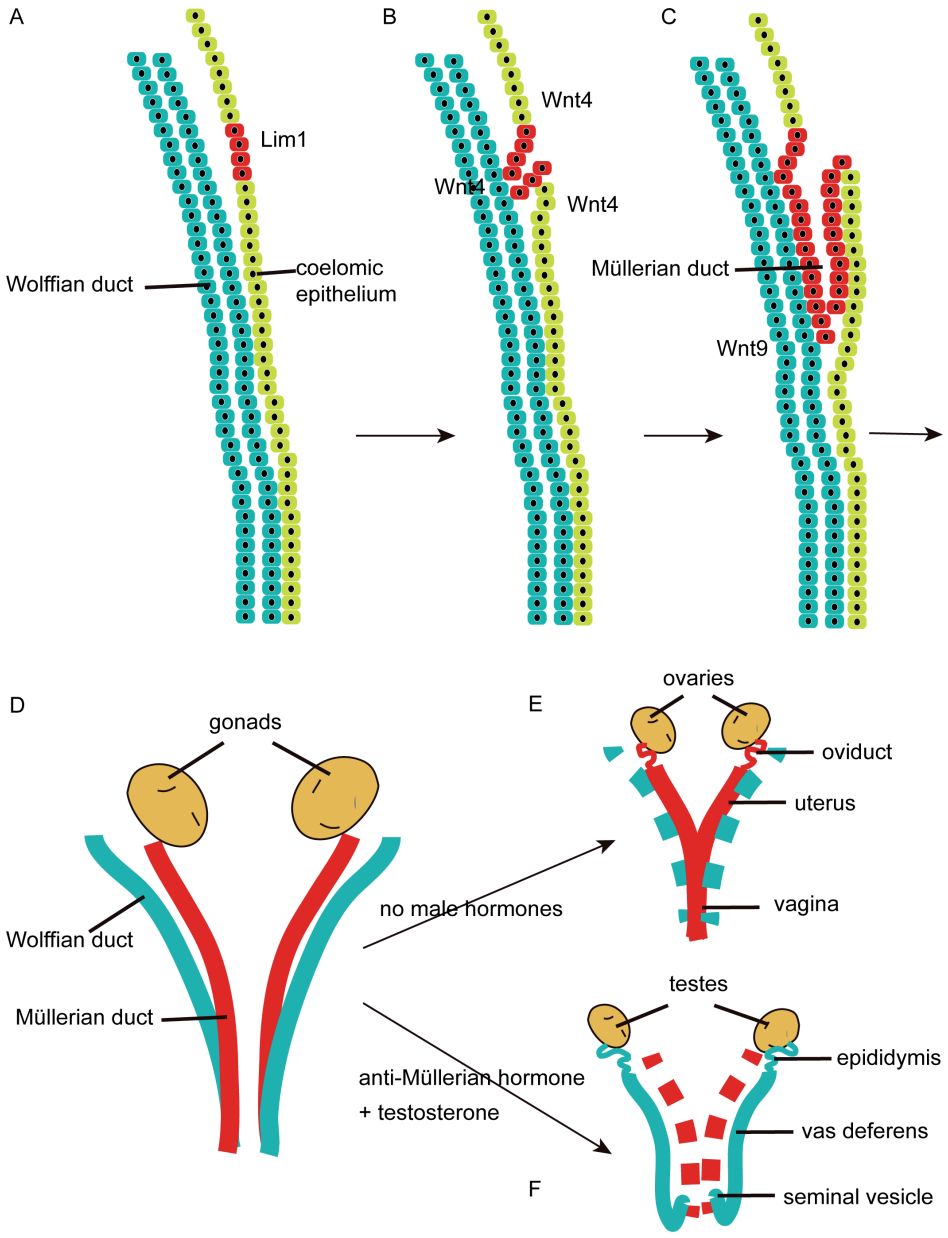

Figure 1. Schematic overview of female reproductive tract development during embryogenesis. Müllerian duct formation (A-C), Wolffian and Müllerian ducts coexist (D), Wolffian duct regression (E), Müllerian duct regression (F). Adapted from Kobayashi and Behringer, 2003.

1.2 ADULT UTERINE PHYSIOLOGY

The female internal reproductive system contains four main parts: the ovaries, oviducts, uterus and vagina (Fig. 2, left: human; right: mouse). The four parts connect to each other and have different functions. The ovaries produce ova and secrete hormones to regulate female physiology. The oviducts, also known as Fallopian tubes, are two tubes linking the ovaries and the uterus. In mice, oviducts are connected to the ovaries by a bursal pouch, which is not present in human. The oviducts are the place where the ovum normally gets fertilized. The uterus is the biggest female internal reproductive organ and it is designed to feed and protect the developing embryo. The vagina is the tract between the uterus and the exterior of the body. It functions as a receptacle for the male's sperm during coitus, a passage for menstrual tissue and blood, and as the birth canal.

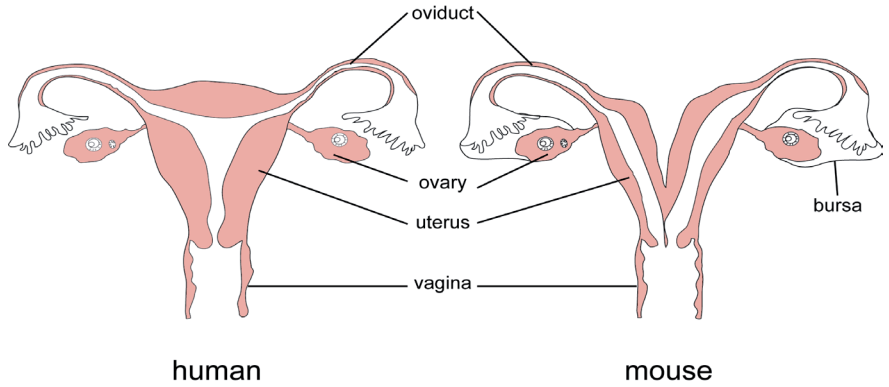

Figure 2. Schematic representation of the human (left) and mouse (right) female reproductive tract. The murine uterus has two horns and the ovaries are covered by bursae.

The inner layer of the human uterus, the endometrium is a dynamic tissue that undergoes hundreds of cycles of proliferation, differentiation and shedding during a woman's reproductive years[14]. The human endometrium can be divided into two layers, the *functionalis* and the *basalis*. Whereas the *functionalis* layer comprises the upper two-thirds of the endometrium and is shedded during menstruation, the *basalis* includes the lower one-third which remains intact and is responsible for producing a new *functionalis* during each subsequent menstrual cycle[14-15].

It is the fine balance between the activities of the two female sex hormones, estradiol and progesterone, which determines lineage faith in the endometrium (Fig. 3A).

During the first two weeks of the human menstrual cycle, thecal cells of the ovary produce large amounts of estradiol. Simultaneously the endometrial estrogen receptor (ER) expression level (mainly ERα) is increased[16]. Upon ligand binding, ER dimers will translocate to the nucleus where they activate transcription of downstream target genes (e.g. insulin-like growth factor; *IGF1*), that stimulate endometrial proliferation[17]. Later, during the third and fourth week of the menstrual cycle, the corpus luteum starts producing progesterone thus inhibiting estradiol-induced proliferation of endometrial cells and stimulating cellular differentiation[18-19]. If no fertilized oocytes are implanted in the uterus, the corpus luteum cannot be maintained due to lack of human chorionic gonadotropin (hCG), and both estrogen and progesterone levels will decline. Withdrawal of progesterone leads to endometrial cell apoptosis and tissue breakdown of the *functionalis* of the endometrium, resulting in its shedding from the uterus [20].

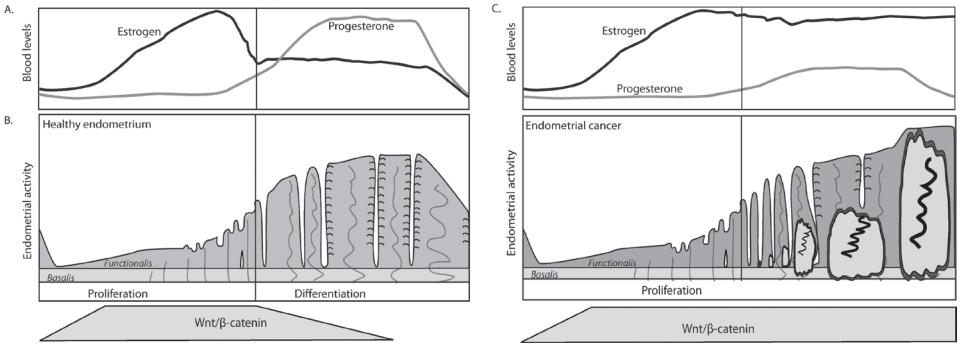

Figure 3. Hormonal (A) and morphological (B) changes during the normal menstrual/estrous cycle in human/mouse and during endometrial oncogenesis (C). In case of enhanced or unopposed estrogen signaling, the endometrium may develop hyperplasia, which may eventually develop further into endometrial cancer. Changes in Wnt/β-catenin signalling in the endometrium are indicated at the bottom of the figure. Adapted from Wang et al. 2010.

1.3 WNT SIGNALLING AND ITS ROLE IN ADULT UTERINE PHYSIOLOGY

Since the discovery of the proto-oncogene *Wnt1* in 1982, the Wnt signalling pathway has been shown to be a key regulator in development, adult homeostasis, and disease [21-22]. Currently, 20 secreted Wnt proteins have been identified among mammalian species that can bind to cell surface receptors of the Frizzled family[22]. Upon binding, three different pathways can be activated: the canonical Wnt/ β-catenin signaling pathway[22], the non-canonical Wnt/Planar cell polarity pathway[23] or the Wnt/Ca2+ pathway[23]. Because in an early stage of our investigations progesterone inhibition of canonical

Wnt/β-catenin signaling was observed, in this thesis, we have focused on canonical Wnt/β-catenin signaling in the female reproductive tract. Central in canonical Wnt/β-catenin signaling is the destruction complex, a multi-protein complex consisting of the scaffold proteins Axin1 and Axin2 (conductin), β-catenin (CTNNB1), the tumor suppressor APC (adenomatosis polyposis coli) and the Ser-Thr kinases CK1 (casein kinase I) and GSK3β (glycogen synthase kinase 3 beta)[24]. In the absence of Wnt ligands, formation of the destruction complex triggers Thr/Ser-phosphorylation of β-catenin by CK1 and GSK3β, and its subsequent ubiquitination and proteasomal degradation. Upon Wnt signaling, the formation of the destruction complex is inhibited thus leading to cytoplasmic accumulation of β-catenin and its nuclear translocation[22]. Once in the nucleus, β-catenin interacts with members of the TCF/LEF transcription factor family, thus regulating the expression of a broad spectrum of Wnt/β-catenin signaling downstream target genes[22,25,26]. The latter include genes encoding proteins with a central role in cell proliferation and survival such as cyclin D1 (*CCND1*) and *MYC*, in addition to roles in a broad spectrum of other cellular functions i.e. cellular migration (e.g. *CD44*), cell adhesion (*CDH1*), extracellular matrix (*MMP7*) and many others[22,27].

The role of Wnt/β-catenin signaling in the regulation of tissue homeostasis has been extensively investigated for the mouse gut[22,28]. Along the mouse intestinal tract, stem cells are located at the bottom of the crypts of Lieberkuhn where they give rise to new stem cells and to proliferating progenitor cells (transient amplifying, TA, cells)[29]. These progenitor cells actively divide and produce new cells that are pushed up along the flank of the crypt towards the villus and eventually differentiate into Goblet cells, enteroendocrine cells and absorptive epithelial cells[30]. A somewhat similar process seems to take place in the endometrium, where estrogen receptor activation in the basalis stimulates endometrial cell proliferation during the first half of the menstrual/estrous cycle, thus giving rise to the *functionalis*. In the 2nd half of the cycle, the corpus luteum will produce progesterone, which reduces estrogen-driven proliferation and induces differentiation of the *functionalis* thus preparing the endometrium for implantation of the fertilized oocyte (Fig. 3B).

Wnt/β-catenin signaling activity along the crypt-villus axis of the intestine follows a decreasing gradient from the stem cell and proliferative compartment to the more differentiated compartment[31]. In the endometrium, Wnt/β-catenin signaling has also been suggested to play a role in regulating proliferation and differentiation during the menstrual cycle. Nei et al. (1999)[32] observed that, in the human endometrium, nuclear β-catenin was clearly enhanced during the proliferative phase of the menstrual cycle, while it was mostly found in the cytoplasm and at the cell membrane during the

secretory phase. Recently, it was also observed that estradiol induces stabilization of intracellular β-catenin in the endometrium, and, upon inhibition of Wnt signaling (by using adenoviral *SFRP2*), estradiol-induced proliferation was abolished[33]. In two other studies[34-35] LiCl was applied to the drinking water of mice to inhibit GSK3β activity and thereby activate Wnt signaling. LiCl-treated animals were characterized by increased proliferation and hyperplasia of the endometrium, thus mimicking sustained estrogen signaling.

In summary, during the menstrual cycle in human and the estrous cycle in mouse, Wnt/β-catenin signaling is important to maintain the balance between proliferation and differentiation. (Fig. 3B)

1.4 INVOLVEMENT OF HORMONE SIGNALING IN UTERINE PATHOPHYSIOLOGY

Besides playing a role in regulation of the menstrual cycle, the sex hormones are also involved in uterine pathophysiology, exemplified in diseases such as endometriosis and endometrial cancer (Fig. 3C).

Endometriosis, a common and benign gynaecological disorder, is characterized 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. Endometriosis has been reported to be an estrogen-dependent disease displaying reduced progesterone receptor levels and resistance to progesterone therapy[36-38].

Endometrial cancer is one of the most common cancers of the female genital tract [39]. It accounts each year for approximately 142.000 new cases diagnosed worldwide, and for 42.000 deaths[40-41]. Endometrial cancer is the seventh most common malignant disorder and its incidence is expected to increase in the near future due to the increase in life span and obesity[42]. Based on epidemiology, conventional histopathology, and clinical behavior, endometrial carcinoma can be divided into two subtypes. Type I endometrial cancer, comprising approximately 85% of the total endometrial carcinoma burden among western societies, resembles normal endometrial hyperplasia in morphology and is associated with increased or unopposed estrogen signaling[41]. Type I endometrial cancer often shows mutations in the *PTEN* and in DNA mismatch repair genes (*MLH1*, *MSH2*, *MSH6*). Also, oncogenic mutations in *KRAS* and/or *CTNNB1* (β-catenin), are recognized major alterations[43-45]. Type II endometrial cancer occurs predominantly in older post-menopausal women, is not correlated to increased estrogen exposure, and is generally associated with a poorer prognosis. Type II endometrial cancers often show

mutations in *P53* and *ERBB2* (Her2/neu)[41,44-45].

In western, industrialized countries, two large groups of women are at increased risk of developing endometrial cancer: (i) women with significant overweight[42], and (ii) those receiving tamoxifen for breast cancer treatment[46-47]. Tamoxifen is a selective estrogen receptor modulator (SERM) acting as an antiestrogen in mammary tissue, but showing estrogenic activity in the endometrium[47]. Currently, it is estimated that up to 40% of all endometrial cancers could be related to obesity[42]. Since the prevalence of obesity is increasing, the incidence of obesity-related endometrial cancer is also on the rise[48]. Additional risk factors for the development of endometrial cancer include: polycystic ovarian syndrome, skipping menstrual periods, being nulliparous, late menopause onset, and the use of unopposed estrogen as hormone substitution therapy[49]. Hence, enhanced estrogen signaling for prolonged periods of time, also as the result of insufficient progesterone levels, represents the main risk factor in endometrial cancer. Progesterone antagonizes the proliferative activity of estrogen by inducing epithelial and stromal cell differentiation in the endometrium [50-51]. In fact, although progesterone (in the form of medroxyprogesterone acetate (MPA)) can be used for the palliative treatment of well-advanced and recurrent endometrial cancer, this treatment has a modest response rate (15-25%)[52]. In contrast, when progesterone, is employed as a primary treatment (e.g. in pre-menopausal women suffering from well-differentiated endometrial cancer and determined to preserve fertility), response rates are considerably improved (up to 60% or more)[53,54].

1.5 WNT SIGNALLING IN UTERINE PATHOPHYSIOLOGY

Gene mutations leading to constitutive activation of canonical Wnt signaling have been found in many different cancer types (e.g. breast, colon, stomach, liver, ovary, skin, etc). Also in the case of endometrial cancer, activation of Wnt/β-catenin signaling is likely to play an important role in early tumorigenesis[43,55]. A substantial fraction of well differentiated endometriod carcinomas (Type I) cases (31%:[56]; 85%:[57] show nuclear β-catenin staining. Accordingly, loss- and gain-of-function mutations in members of the Wnt/β-catenin signaling pathway known to act as tumor suppressors (APC) and oncogenes (β-catenin) have also been identified. Mutation of β-catenin at its GSK-3β binding consensus site encoded by exon 3 and leading to its constitutive activation have been identified in 15-40 % of endometrial tumors[58,59], whereas loss of heterozygosity (LOH) at the *APC* locus was observed in 24 % of the cases with nuclear β-catenin staining[60]. *APC* mutation analysis showed truncating mutations in 10% of all endometrial cancers[61].

Moreover, the *APC* A1 promoter was found to be hypermethylated in 46.6% of endometrial cancers positive for nuclear β-catenin[60], often in correlation with microsatellite instability[62]. Notably, these somatic mutations in members of the canonical Wnt pathway were preferentially found in Type I endometrial cancer, which accounts for about 85% of the total number of endometrial cancer cases. Enhanced estrogen signaling over a prolonged period of time is believed to be a causative factor for Type I endometrial cancer[63]. Hence, although continuous estrogen induced Wnt/β-catenin signaling may represent an early step in endometrial tumorigenesis, tumor progression and malignant transformation seem to additionally require somatic mutations leading to the constitutive activation of the pathway.

Several genetically engineered mouse models have been employed to investigate the role of constitutive Wnt/β-catenin activation for endometrial malignancies. Tanwar et al. (2009)[64] investigated the effects of conditional Wnt signaling activation in the uterus by *Amhr2-Cre*-driven oncogenic activation of β-catenin (*Ctnnb1*$^{tm1Mmt/+}$). The *Amhr2* gene is expressed as of mouse embryonic day 12.5 onwards in mesenchymal cells surrounding the Müllerian duct and in adult mice in the myometrium. The corresponding *Amhr2*$^{Cre/+}$*;Ctnnb1*$^{tm1Mmt/+}$ mice develop myometrial hyperplasia and mesenchymal tumors (similar to leiomyomas) and endometrial sarcomas. Furthermore, hyperplasia of endometrial glands was occasionally observed in the uterus, suggesting that mesenchymal activation of Wnt/β-catenin signaling plays a role in the early events of epithelial tumorigenesis in the endometrium. Jeong et al. (2009)[65] employed *Pgr-Cre* to drive oncogenic activation of β-catenin (*Pgr*$^{Cre/+}$*;Ctnnb1*$^{f(Ex3)/+}$) and of canonical Wnt signaling in a broad spectrum of uterine cells (endometrium and myometrium). These authors could show that activation of Wnt/β-catenin signaling in the uterus resulted in enhanced proliferation of glandular epithelial cells, endometrial hyperplasia at 6 weeks of age, and in defective estrogen signaling, though not in endometrial cancer. These results suggest that constitutive activation of Wnt/β-catenin signaling on its own is insufficient for endometrial cancer onset and may possibly also indicate that the synergistic action of additional downstream effectors of estrogen signaling are necessary for full-blown malignant transformation. However, as predicted by the just-right signaling model[66-67], different levels of pathway activation may differently trigger tumorigenesis in distinct tissues. Therefore, it would be of interest to investigate the consequences of hypomorphic mutations in members of the Wnt pathway other than β-catenin whose oncogenic activation invariably leads to extremely high Wnt signaling levels.

1.6 THE RELATIONSHIP BETWEEN SEX HORMONE ACTION AND WNT/B-CATENIN SIGNALING IN THE ENDOMETRIUM

Signaling by both sex hormones and Wnt/β-catenin are actively involved in endometrial physiology and pathophysiology, and there are good indications that during the menstrual cycle, sex hormones can modulate Wnt/β-catenin signaling to maintain the balance between proliferation and differentiation (Fig. 3).

1.6.1 Estrogenic regulation of Wnt/β-catenin signaling.

The putative mechanisms underlying estrogen-mediated Wnt/β-catenin activation in the uterus are at present poorly understood. A direct effect of ERα as an estrogen-activated transcription factor modulating the expression of Wnt ligands, modulators and targets has been described by many authors: the ligands Wnt4, Wnt5A and Wnt7A have been shown to be induced by estradiol in the uterus[33,68-71]. In addition the Wnt inhibitor DKK1 was shown to be inhibited by estrogens in bone forming osteoblasts[72] and in the hippocampal CA1 region[73] (DKK1 interacts with a co-receptor for canonical Wnt signaling, the low density lipoprotein receptor-related protein-6 (LRP6)[74]). Furthermore, the Wnt-target gene *WISP2* (Wnt1-induced signaling pathway protein 2) was shown to be up-regulated through direct interaction of activated ERα with its promoter region in human breast cancer cells[75].

The estrogen-activated ERα, however, can also function as a modulator of transcription, without directly binding to DNA sequences in the promoter region of the affected genes. For example, Shi et al. (2007)[77] could show that EZH2 (the polycomb group protein enhancer of zeste homolog 2) physically interacts with ERα and β-catenin. Hence, estradiol can affect the transcription of Wnt/β-catenin target genes without directly interacting with estrogen response elements at the DNA level.

Furthermore, ERα has also been observed to associate with important growth factor pathways such as the PI3K pathway thus indirectly cross-talking with canonical Wnt signaling[78,79]. Binding of estrogen-activated ERα to p85 (PIK3R2), the regulatory subunit of PI3K, activates AKT which results in inhibition of GSK-3β. This, in turn, prevents N-terminal Ser/Thr phosphorylation of β-catenin, enhances its intracellular stabilization and eventual allows for translocation to the nucleus, where it complexes with members of the TCF/LEF transcription factor family and leads to the activation of Wnt target genes[80,81] (Fig. 4A and Fig. 3).

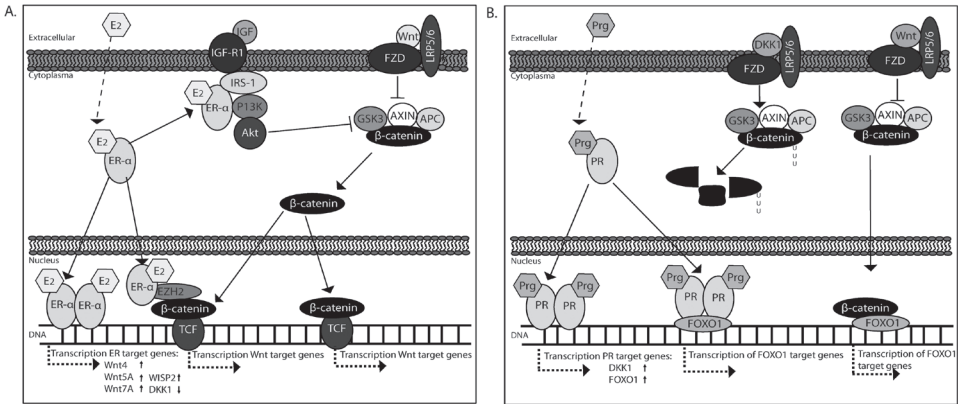

Figure 4. Mechanisms of estrogenic (A) and progestagenic (B) regulation of Wnt/β-catenin signaling in the human or mouse endometrium. Adapted from [76]. Akt, protein kinase B; E2, estradiol; EZH2, enhancer of zeste homolog 2; FZD, Frizzled receptors; IRS-1, Insulin receptor substrate 1; LRP, low-density lipoprotein receptor related protein; Prg, progesterone; TCF, T-cell factor.

1.6.2 Progestagenic regulation of Wnt/β-catenin signaling

A role for progesterone in regulating Wnt/β-catenin signaling was suggested by Kao and Elinson (1998)[82] and Tulac et al. (2006)[83] who observed a profound progesterone specific increase in expression of the Wnt/β-catenin signaling inhibitor DKK1 in endometrial stroma cells during the secretory phase of the menstrual cycle. Using the antiprogestagen mifepristone (RU486), Catalano et al. (2007)[84] could indeed confirm progestagen regulation of many Wnt/β-catenin pathway components. Furthermore, Kane at al. (2008)[85] subsequently showed that TGFβ1 attenuates both the expression of PR and DKK1 in differentiated endometrial stromal cells corroborating a close link between progesterone and Wnt/β-catenin signaling. FOXO1 is also an interesting molecule, as it has been shown to physically interact with the progesterone receptor to coordinate cell cycle regulation and differentiation of human endometrial stromal cells[86]. Furthermore, FOXO1 is also able to interact with β-catenin[87-88], thus being able to directly inhibit Wnt/β-catenin signaling[89].

Another pathway likely to play a role in regulating the interplay between progesterone and Wnt/β-catenin signaling is the hedgehog signaling pathway. Combined endometrial microarray data from different stages of the menstrual cycle[17] and from E2 only or E2+MPA treated patients[51] indicated profound sex hormone regulation of Hedgehog signaling. Mammals have three different Hedgehogs, Sonic (SHH), Indian (IHH), and Desert (DHH). The IHH, the common Hedgehog receptor patched (PTCH), and the target gene *GLI1* encoding the transcription factor GLI1 that is implicated in the hedgehog signaling pathway, are all up-regulated upon

estrogen signaling and down-regulated by progesterone during the menstrual cycle. Furthermore, it has been shown that when IHH expression is impaired, the downstream effects of progesterone are lost in the uterus[90]. As indicated, progesterone itself down-regulates Hedgehog signaling in the uterus and it has been shown that Wnt/β-catenin signaling may act downstream to IHH signaling[91].

Recently the link between Hedgehog and Wnt/β-catenin signaling has also been confirmed for atypical endometrial hyperplasia and endometrial cancer, showing that in hyperplasia and in well differentiated endometrial cancers GLI1 overexpression correlated well with β-catenin nuclear immunoreactivity[92]. Because our own data on staining for the Wnt target gene *CD44* indicated that progesterone can also act as a profound inhibitor of Wnt/β-catenin signaling *in vivo* in hyperplasia as well as in well differentiated endometrial cancer, a physiological and functional link between progesterone, hedgehog and Wnt/β-catenin signaling seems plausible. (Fig. 4B and Fig. 3)

1.7 STEM CELLS OF THE ENDOMETRIUM

As briefly indicated above, the endometrium is a very dynamic tissue, which, in human, is partly replaced every 4 weeks, and in mice every 4 to 5 days. For other tissues with significant cellular turnover, such as the hematopoietic system, the epidermis and intestinal epithelium, somatic stem cells have been identified[93-95]. However, although the concept of stem or progenitor cells replenishing the endometrium was proposed three decades ago[96], until now, direct evidence showing that the endometrium does have its own stem/progenitor cells is limited.

Chan et al. (2004) showed that, by plating out single human endometrial cells, 0.08% of endometrial epithelial cells and 0.02% of stromal cells formed large colonies which could be replated several times[97]. Epithelial markers, such as BerEP4, CD49f and cytokeratin, and stromal markers such as 5B5, αSMA and CD90 could be identified in epithelial and stromal colonies, respectively. Further, Gargett et al. (2009) showed that epithelial colonies in matrigel could form cytokeratin expressing glandular structures. In addition depending on the differentiation-induction medium, stromal colonies showed differentiation into the myogenic lineage (αSMA and caldesmon staining), osteogenic lineage (alkaline phosphatase reactivity and expression of PTHR1), adipogenic lineage (Oil Red O and lipoprotein lipase (LPL) staining) and chondrogenic lineage (alcian blue and collagen type II staining)[98]. Other research on the identification of endometrial stem cells involved an interesting finding that in bone-marrow transplanted patients,

donor-derived bone marrow cells could be found in the endometrial epithelium and stroma[99]. This experiment was confirmed in lethally irradiated female mice that were transplanted with bone marrow derived from constitutively LacZ expressing male mice, showing > 0.5% of epithelial endometrial cells originating from the male donor[100, 101].

The above-mentioned studies, aimed at the identification of stem/progenitor cells, all suffer from a major drawback that although stem/progenitor cells seem to be present in the endometrium, these cells cannot be recognized as such in their original niche, neither can they be specifically isolated for further research.

One approach to identify and isolate stem-like cells is by sorting by FACS a population of cells on the basis of low staining by the fluorescent vital DNA binding dye, Hoechst 33342[102]. These cells are called side population cells (SP cells), and due to high expression of ABCG2/Bcrp1 transporter proteins, stem-like cells actively remove Hoechst 33342 from their cytoplasm[103]. This characteristic was used to isolate cancer stem cells in several malignancies including acute myelogenous leukemia, neuroblastoma, glioma and lung cancer[104-108]. For the human endometrium Tsuji et al. (2008) isolated SP cells that were initially in the G_0 phase of the cell cycle and which showed greater colony forming efficiency than non-SP cells[109]. The fact that isolated SP cells were in G_0 is reminiscent of the common suggestion that stem cells are a relatively quiescent cell population. Furthermore, under decidualizing conditions colony forming endometrial SP cells started to secrete prolactin which is in agreement with the receptive nature of the endometrium towards an implanting embryo. Cervello et al. (2010) went a step further and showed in vitro differentiation of SP cells into adipogenic and osteogenic lineages. Moreover, after subcutaneous injection of SP cells in NOD-SCID mice, these authors observed the development of human endometrium-like structures[110].

Another approach to find stem cells in the endometrium is by staining the tissue for known stem cell markers. For example, recently a population of single cells and cell groups in the stroma adjacent to the myometrium was identified that expressed the putative stem cell marker Musashi-1[111]. Interestingly this marker was also found to be expressed at increased levels in endometriosis and endometrial carcinoma[112].

Taking advantage of the finding that stem cells may be infrequently dividing [113], two methods have been developed to identify these cells on the basis of label retention (label retaining cells; LRCs). Mice are labeled using a traceable exogenous DNA marker such as bromodeoxyuridine (BrdU)[114,115] or using an inducible hybrid DNA binding transgene such as histone-2B GFP (H2B-GFP). Subsequently, in each cell division the label is washed-out except for in non- or infrequently-dividing stem-like cells. Using

BrdU labeling Gargett[116] identified slowly dividing cells in the endometrium. They observed that after 12 weeks of chase, no LRCs were present in the luminal and glandular epithelium, and stromal LRCs were observed at the endometrial-myometrial junction in close proximity to the blood vessels. At 8 weeks of chase, however, approximately 3% of endometrial epithelial cells still retained their label. These ER-negative LRCs were, in contrast to the authors expectations, present in the luminal epithelium.

The inducible H2B-GFP system to label DNA has been used with great success to identify stem cells in the hematopoietic system[117-119], hair follicles[120], and rectum[121] , but has not been used before, to study the mouse endometrium.

1.8 OUTLINE OF THIS THESIS

Cyclic renewal and functional differentiation of the inner layer of the uterus depends on hormonal regulation of a group of cells which is able to rapidly repopulate the functionalis layer of the endometrium. The cells which are at the origin of the repopulation, endometrial progenitor cells, are induced to proliferate by estradiol and induced to differentiate by progesterone.

In **Chapter 1** the importance of sex hormonal and Wnt signaling in endometrial homeostasis and tumorigenesis is addressed. To further the role of Wnt/βcatenin signaling in more detail, and in particular to study what is the effect of loss of APC expression in the uterus, we conditionally inactivate the endogenous Apc gene from early embryonic life onwards in mesenchymal cells surrounding the Müllerian duct. The results are presented in **Chapter 2**.

As described in **Chapter 3**, we investigated how progesterone counteracts the proliferative effects of E2 during the normal menstrual cycle, during hyperplasia, and during early endometrial carcinogenesis. The molecular mechanistic evidence is provided.

The research described in **Chapter 4** focused on the question whether we could identify stem cells in the female urogenital tract. To answer this question, we have employed a non-mutagenic and cell cycle independent approach, namely in vivo pulse-chase with the histone 2B - green fluorescent protein (H2B-GFP) towards the identification and prospective isolation of long-term LRCs (LT-LRCs) in the mouse female reproductive tract. The characteristics of the LT-LRCs were described.

Chapter 5 and 6 provides a summary of the main findings of the present research. This includes a discussion of the significance of the findings and the potential future perspectives.

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2

LOSS OF APC FUNCTION IN MESENCHYMAL CELLS SURROUNDING THE MÜLLERIAN DUCT LEADS TO MYOMETRIAL DEFECTS IN ADULT MICE

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ABSTRACT

The Wnt signal transduction pathway plays a rate limiting role in early development of many different organs. To study the functional consequences of constitutive activation of the canonical Wnt pathway in the developing uterus, we generated a novel mouse model where loss of the tumor suppressor gene *Apc* was induced. A mouse model was generated and evaluated where *Amhr2*$^{Cre/+}$ driven loss of *Apc exon 15* was induced. The *Apc* recombination was detected mainly in the myometrial layer of the adult uterus. A significant loss of muscle fibers in myometrium was apparent, though with very few muscle cells earmarked by nuclear β-catenin. The finding was confirmed in the *Pgr*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mouse model. Loss of APC function in mesenchymal cells surrounding the fetal Müllerian ducts results in severe defects in the myometrial layers of the uterus in adult mice, suggesting that the WNT signaling pathway plays important roles in maintaining myometrial integrity.

INTRODUCTION

Shortly after the onset of testis differentiation during mammalian embryonic life, the first testicular hormone to be produced is anti-Müllerian hormone (AMH), secreted by the Sertoli cells, soon followed by production of testosterone by the Leydig cells. Fetal testosterone production stabilizes the Wolffian ducts, which give rise to the epididymides and vasa deferens. AMH binds to its receptors on the mesenchymal cells surrounding the Müllerian ducts, which induces apoptosis and results in Müllerian duct regression. Because embryonic female gonads do not produce any testosterone or AMH, Wolffian ducts will regress and Müllerian ducts are stabilized to form the upper part of the vagina, the uterus and the oviducts[1]. For the current investigations, we developed and analysed a novel mouse model to study the role of canonical Wnt/β-catenin signalling in embryonic development and homeostasis of the uterus.

In canonical Wnt/β-catenin signalling, a central role is played by the "destruction complex" consisting of three scaffolding proteins, namely AXIN1, AXIN2, and APC (adenomatous polyposis coli), two kinases, glycogen synthase kinase-3β (GSK3β) and casein-kinase 1α (CK1α), and β-catenin (CTNNB1). In the absence of extracellular Wnt ligands, formation of the destruction complex marks β-catenin by Ser-Thr phosphorylation, thus promoting its proteolytic degradation through ubiquitination. In the presence of Wnt signals however, formation of the destruction complex is inhibited and β-catenin is able to accumulate in the cytoplasm and eventually translocate to the nucleus where, in association with members of the TCF/LEF family of transcription factors, it activates transcription of a broad spectrum of downstream target genes (http://www.stanford.edu/~rnusse/pathways/targets.html). Defects in several members of the Wnt signalling cascade, i.e. loss of function mutations at the *APC* gene, result in the accumulation of intracellular and nuclear β-catenin, thus leading to constitutive deregulation of target genes[2-6]. During the early phases of development of the female reproductive tract, Wnt ligands such as Wnt4, Wnt5a and Wnt7a play an important role[7-9]. *Wnt4* knock-out female mice develop normal Wolffian ducts but lack Müllerian ducts[9]. *Wnt5a* knock-out female mice are characterized by defects in the posterior outgrowth of the developing Müllerian ducts and in forming endometrial glands[7]. Loss of Wnt7a expression on its turn, leads to incomplete demarcation between the vagina and the uterus and between the uterus and the oviduct. Furthermore, *Wnt7a* knock-out female mice do not form uterine glands and show myometrial aberrations[8].

More recently, two models have been developed to evaluate the consequences of conditional Wnt/β-catenin activation in the uterus[10-11]. Jeong et al. (2009) investigated the effects of conditional activation of Wnt signaling in the uterus by *PgrCre* driven activation of β-catenin expression (*Pgr*$^{Cre/+}$;*Ctnnb1*$^{f(Ex3)/+}$) and showed profound endometrial hyperplasia, which did not further progress to endometrial cancer [10]. Tanwar et al. (2009) employed the *Amhr2* (Amh type 2 receptor) promoter to drive Cre expression (*Amhr2*$^{Cre/+}$) and activate β-catenin expression in mesenchymal cells surrounding the developing Müllerian ducts. This approach resulted in myometrial hyperplasia, adenomyosis, and mesenchymal tumors similar to leiomyomas and endometrial stromal sarcomas[11]. Furthermore, hyperplasia of endometrial glands was occasionally observed suggesting that mesenchymal activation of Wnt/β-catenin signalling plays a role in the early events which may lead to endometrial carcinogenesis.

In approximately 40% of human endometrial cancers, nuclear β-catenin staining is observed[12-13], and in over 50% of these cases loss of APC expression is involved (either LOH or promoter methylation[14]). In order to further clarify the role of Wnt/β-catenin signalling, and in particular loss of Apc expression in the uterus, we bred *Amhr2*$^{Cre/+}$mice[15] with *Apc*$^{15lox/15lox}$ mice[16] to conditionally inactivate the endogenous *Apc* gene from early embryonal life onwards in mesenchymal cells surrounding the Müllerian duct.

MATERIALS AND METHODS

Animals and genotyping

All experiments conducted with mice were approved by our local animal ethics committee (DEC permit number EUR 1076) and were in accordance to international guidelines and regulations. *Amhr2*-LacZ animals[17-18] and *Amhr2Cre* animals[15] were obtained from Dr RR Behringer. *Amhr2Cre* mice were bred with Rosa26 reporter mice [19] to review Cre expression, and were bred with *Apc*^{15lox} mice[16,20] to obtain *Apc*$^{15flox/15flox}$ mice ("flox" means that the sequence between two loxP sites has been deleted by Cre). *PgrCre* animals[21] were also bred with *Apc*^{15lox} mice to obtain *Apc*$^{15flox/15flox}$ mice. All animals were maintained in a C57Bl/6J background. Genotyping was performed by routine PCR, on tail and uterus DNA, using the following primers: detection of the 380 bp Apc^{15lox} allele: TAGGCACTGGACATAAGGGC and GTAACTGTCAAGAATCAATGG; detection of the 450 bp Apc^{15flox} allele: AACTTCTGAGTATGATGGAGG and GTAACTGTCAAGAATCAATGG; detection of the 219 bp Cre allele: GGACATGTTCAGGGATCGCCAGGC and CGACGATGAAGCATGTTTAGCTG; detection of the 500 bp Rosa26-LacZ^{lox} and 250 bp Rosa26-LacZ^{flox} allele: AAAGTCGCTCTGAGTTGTTAT, GCGAAGAGTTTGTCCTCAACC and GGAGCGGGAGAAATGGATATG. For all animals, the stage of the estrous cycle was determined by a vaginal smear just before sacrifice.

β-Galactosidase staining

The β-galactosidase staining was performed as described before[22] with the adaptation that here Bluo-Gal (Invitrogen, Breda, The Netherlands) was used instead of X-gal. Fixed uteri were stained with Bluo-Gal overnight, embedded in paraffin, sectioned (5 µm) and counter-stained with eosin.

Laser microdissection

Paraffin-embedded tissues were sectioned (10 µm), adhered to uncoated microscope slides and stained with haematoxylin and eosin. Laser microdissection was performed by PALM laser microdissection (Carl Zeiss MicroImaging GmbH, Standort Göttingen, Germany). Approximately 1 mm^2 of myometrium, luminal epithelium and stromal tissue combined with glandular epithelium were collected, respectively. DNA was extracted from these tissues compartments, using a phenol-chloroform method. The genotypes of different uterine layers were assessed by PCR as described above.

Immunohistochemistry

Immunohistochemistry was performed essentially as described before[23]. The antibodies employed are: mouse monoclonal anti-SMA (DAKO, Heverlee, Belgium) (1:150), rat monoclonal anti-cytokeratine 8 (CK8, 1:5000) (DSHB, Iowa, USA), rabbit polyclonal anti-ERα (Millipore, Billerica, USA) (1:2000) and rabbit monoclonal anti-β-catenin (Epitomics, Burlingame, USA) (1:800).

Histological view and measurement

Immunohistochemical staining for smooth muscle actin (SMA), which stains myometrium, was applied to distinguish between myometrium and endometrium. CK8 was used to specifically stain epithelial cells. Nanozoomer Digital Pathology (NDP) (Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany) was used to scan the slides and this program also allowed for uterine area measurements. The number of glands was measured per square millimeter of endometrium. Of each evaluated uterus, every 2mm one section was measured (7 sections per uterus), and in total thirteen control and ten experimental animals were assessed.

Statistical methods

Analyses were performed using Graphpad Prism 5 (GraphPad Software, Inc., San Diego, CA). Unpaired, two-tailed t-test was used to analyze the differences between groups. P values lower than 0.05 were considered statistically significant.

RESULTS

2

The Amhr2 promoter is mainly active in the myometrium

First we established the pattern of expression driven by the promoter of *Amhr2* in the uterus by employing previously developed *Amhr2*-LacZ reporter mice[17]. As shown in Figure 1A, LacZ expression was limited to the myometrial layer of the uterus. This result was confirmed by breeding *Amhr2*$^{Cre/+}$ transgenic animals with the Rosa26 reporter mice (R26R) carrying a loxP-STOP-loxP-lacZ reporter[19]. In the presence of active Cre, somatic deletion of the STOP cassette is achieved, thus leading to β-galatosidase expression from the *LacZ* gene. Figure 1B shows β-galatosidase staining of both myometrial layers (inner circular and outer longitudinal muscles) in compound *Amhr2*$^{Cre/+}$;R26R animals. Notably, staining was not continuous throughout the myometrium as exemplified by patchy β-galactosidase expression within the outer myometrial layer, which is in accordance with a previous publication by Deutscher and Yao[24]. Staining for β-galactosidase also indicated that the *Amhr2* promoter is not active in stromal and epithelial cells of the endometriumNext, *Amhr2Cre* animals were bred with *Apc*$^{15lox/15lox}$ animals, a model developed by our laboratory[16] to conditionally inactivate Apc function by removing exon 15 of the endogenous gene. Deletion of both *Apc* alleles results in the constitutive activation of Wnt/β-catenin signaling. To substantiate that Cre expression in the compound *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals was limited to the myometrial layer, and to verify that recombination of the *Apc* gene was indeed taking effect, laser capture microdissection (LCM) of different layers of the uterus was performed. As illustrated in Figure 1C and 1D, only a small percentage of cells from the stromal/glandular region showed *Apc* exon 15 deletion, whereas no somatic recombination was detected in the luminal epithelial region. In contrast, *Apc* deletion was evident in cells from in the myometrium.

Next, Amhr2Cre animals were bred with *Apc*$^{15lox/15lox}$ animals, a model developed by our laboratory 16 to conditionally inactivate Apc function by removing exon 15 of the endogenous gene. Deletion of both Apc alleles results in the constitutive activation of Wnt/β-catenin signaling. To substantiate that Cre expression in the compound Amhr2$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals was limited to the myometrial layer, and to verify that recombination of the Apc gene was indeed taking effect, laser capture microdissection (LCM) of different layers of the uterus was performed. As illustrated in Figure 1C and 1D, only a small percentage of cells from the stromal/glandular region showed Apc exon

15 deletion, whereas no somatic recombination was detected in the luminal epithelial region. In contrast, Apc deletion was evident in cells from in the myometrium.

Somatic *Apc* deletion is predicted to result in the constitutive activation of the canonical Wnt pathway and, possibly, in the nuclear accumulation of β-catenin[16,25]. However, IHC analysis of β-catenin expression in the uterus of *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals revealed that the majority of myometrial cells are marked by cytoplasmatic β-catenin staining with only few cells showing nuclear staining (Fig. 1E). This result is in agreement with previous studies from our and other laboratories showing that loss of Apc function is necessary but often insufficient for nuclear β-catenin accumulation[26,27].

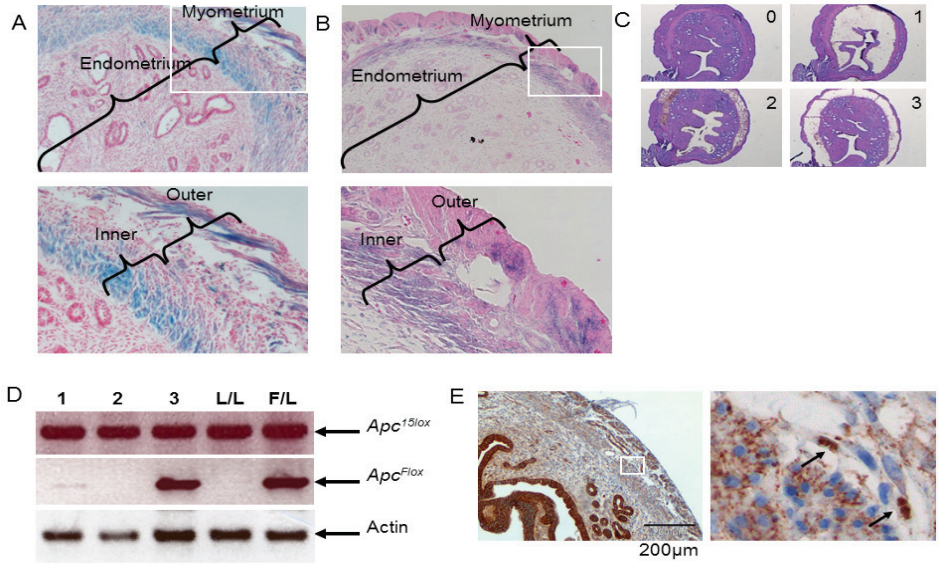


Figure 1. *Amhr2* promoter activity and *Apc* recombination in different regions of the uterus. **A**: *Amhr2-LacZ* mice were stained for β-galactosidase activity with Bluo-Gal. **B**: *Amhr2*$^{Cre/+}$;*R26R* animals were stained for β-galactosidase activity. **C**: Indicated different layers of the uterus that were isolated by microdissection for DNA isolation. (0), before dissection; (1), glands and stroma; (2), luminal epithelium; (3), myometrium. **D**: Representative gel-image from a PCR reaction to determine recombination of *Apc* using DNA isolated from an *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mouse: (1), stroma and glands; (2) luminal epithelium; (3) myometrium. *Apc*^{15lox} represents the non-recombined lox allele; *Apc*^{flox} indicates recombination of *Apc*; Actin was used as a control for PCR. L/L represents DNA displaying both non-recombined *Apc*^{15Lox} alleles; F/L represents DNA displaying one non-recombined and one-recombined *Apc* allele. Primers used for the PCR are described in Materials and Methods. **E**: β-catenin staining of a representative *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mouse. The arrows indicate myometrial cells which show nuclear β-catenin staining. Luminal and glandular epithelial cells only show cytoplasmic and membrane β-catenin staining. Control animals never showed nuclear β-catenin staining.

Loss of APC function results in myometrial defects

Histological assessment of uteri from adult compound *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice revealed severe defects in the myometrium (an overview of the analyzed mice is presented in Table 1). To investigate the nature of these defects in more detail, smooth muscle actin (SMA) IHC staining was employed to specifically mark the myometrium. As shown in Figure 2, in contrast to control animals (Fig. 2A), all *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals showed regions within the myometrium where the muscle layer appeared disorganized or even discontinuous (Fig. 2B). Out of the 19 *Amhr2*$^{cre/+}$;*Apc*$^{15lox/15lox}$ mice here examined, 16 showed tissue architectural changes of the myometrium without interruption of the myometrium layer, as exemplified in Figure 2B (top panel). In the other 3 of 19 animals the same architectural changes in the myometrium appeared in association with a discontinuous myometrium layer, as exemplified in panels 2 and 3 of Figure 2B. Pregnancies in *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals only resulted in life born puppies in 2/4 cases. On the other 2/4 cases the mother died during delivery. In order to investigate these delivery problems futher, we tried to obtain permission with our local animal ethics committee, but it was not allowed.

Table 1: Animals investigated

Genotype	N	Age (weeks)	Phenotype
Wild type	21	7-31	normal
Apc$^{15lox/+}$	9	9-33	normal
Apc$^{15lox/15lox}$	5	8-34	normal
Amhr2$^{cre/+}$	3	7-34	normal
Amhr2$^{cre/+}$;*Apc*$^{15lox/15lox}$	19	8-34	Impared myometrial architecture in 19/19 animals. Discontinuation of the myometrial layer in 3/19 animals.

To obtain additional evidence that loss of Apc function in myometrial cells of the uterus indeed results in muscular defects in the adult uterus, *Apc*$^{15lox/15lox}$ animals were bred with *PgrCre* animals. In these animals nuclear β–catenin staining was readily detected in the myometrium and in epithelial cells of the endometrium (Fig. 3A).

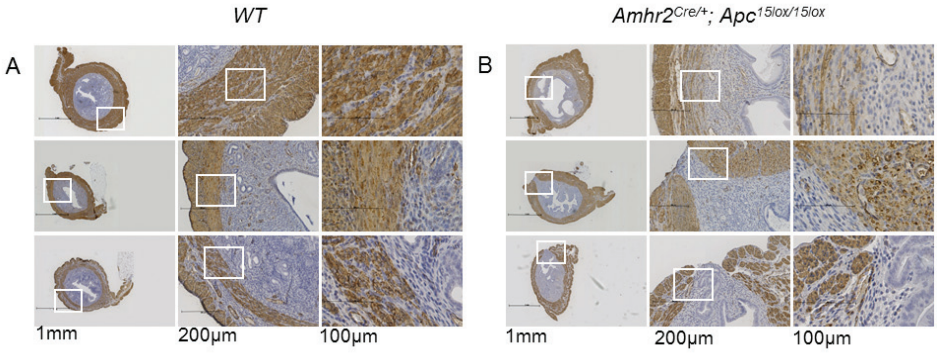


Figure 2. *Amhr2*$^{Cre/+}$*;Apc*$^{15lox/15lox}$ mice show myometrial defects. The uterine muscle was stained with an antibody against Smooth Muscle Actin (SMA) **A**: three control animals at different stages of the estrous cycle: from top to bottom: proestrus, estrus and diestrus. The staining shows a cellular myometrium with closely-packed muscle fibers. **B**: Three *Amhr2*$^{Cre/+}$*;Apc*$^{15lox/15lox}$ mice, matched for age and stage of the cycle to the controls. The top figure shows an affected uterus where the outer part of the myometrium contains tightly packed fibers, but the inner part shows more dispersed and thinner fibers. The middle and bottom figures show a complete trans-section of the uterus where the muscle wall is focally incomplete.

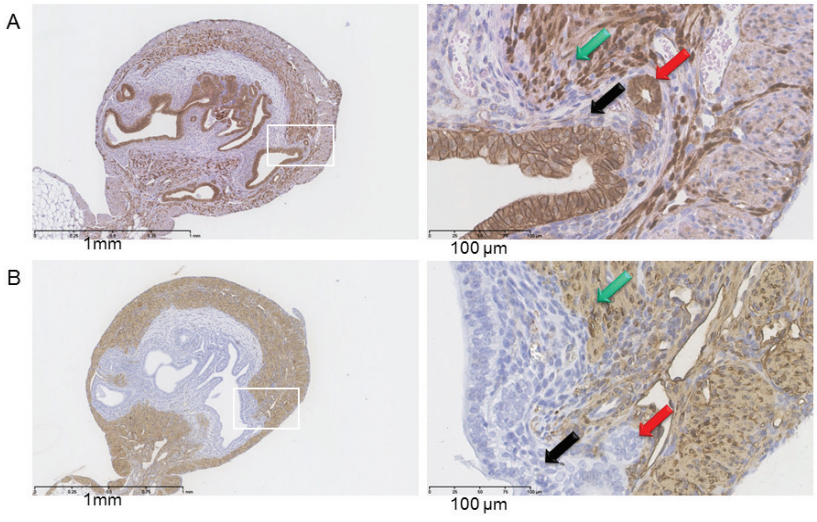


Figure 3. *Pgr*$^{Cre/+}$*;Apc*$^{15lox/15lox}$ mice also display myometrial defects. β-catenin (A) and SMA (B) staining of a representative *Pgr*$^{Cre/+}$*;Apc*$^{15lox/15lox}$ mouse. Details on the right indicate presence of glandular and stromal cells in the muscle layer. Gland (red arrow), stroma (black arrow) and myometrium (green arrow).

Furthermore, marked myometrial defects were detected in the majority of investigated animals (7/10) (Fig. 3B). Interestingly, omissions in the myometrial layer were invaded by stromal and glandular cells from the endometrium reminiscent for a human disorder described as endometriosis interna or adenomyosis[28].

In order to assess the underlying basis for the effects of the induced Apc mutation, embryonal mice were sacrified and the Müllerian ducts were stained for apoptosis (caspase-3). Time of sacrifice (E12) was chosen based on our own data in *Amhr2*$^{Cre/+}$; R26R-LacZ animals showing clear Cre activity around that day. No differences, however, were detected between controls and *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ littermates (data not shown).

Endometrial defects in Amhr2$^{cre/+}$;Apc$^{15lox/15lox}$ mice

Although recombination of *Apc*^{15lox} alleles in the endometrium could hardly be detected in the *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals, phenotypic expression of gene deletion was apparent from observed differences between targeted and control animals, such as a smaller endometrial area and less endometrial glands. To quantify these differences, anti-SMA was used to stain myometrium, and anti-CK8 was used to stain endometrial glands (Fig. 4A and 4B). As indicated in Materials and Methods several areas were measured and gland numbers counted. Despite the myometrial defects described above, no significant differences could be detected in the myometrial area of *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals when compared with control animals (Fig. 4C). However, significant differences were observed for the number of glands per mm^2 (Fig. 4D, $p = 0.0001$) and the area of the endometrium (Fig. 4E, $p = 0.0002$): *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice show fewer glands and a reduction of the endometrial area.

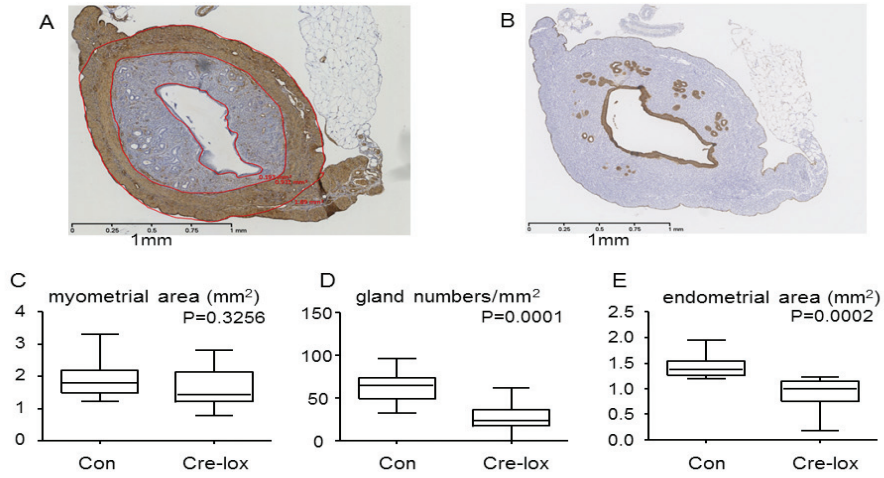
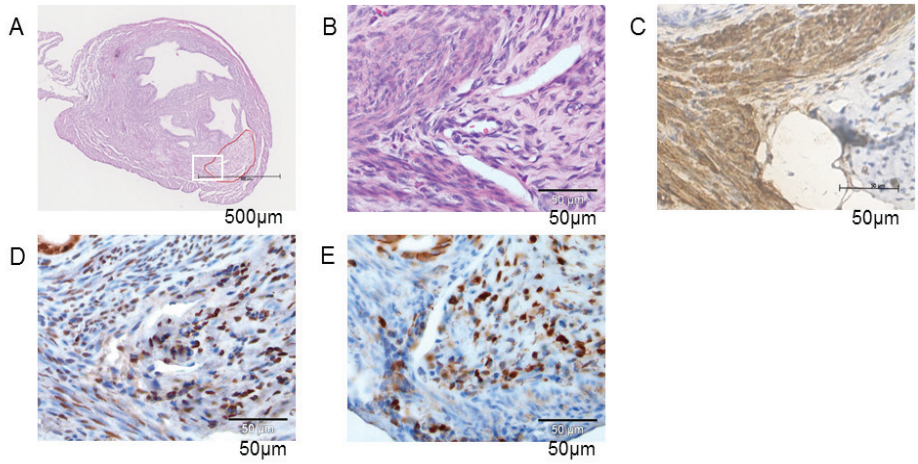


Figure 4. Quantification of endometrial and myometrial defects in *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice. **A**: a cross section from a control uterus stained for smooth muscle actin and counterstained with hematoxylin. Indicated regions provide information on the area of the myometrium and endometrium. **B**: consecutive section was stained for cytokeratine 8 in order to measure the number of endometrial glands. **C**: calculation of the myometrial area in control and affected animals **D**: measurement of the number of glands per area of stroma in control and affected animals. **E**: calculation of the endometrial area in control and affected animals. The panels **C-E** were derived from 13 controls and 10 *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice (7 sections per animal). Con = control animals; Cre-lox = *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals. Data are presented as: Mean (line), 25th and 75th percentiles (box), and 5th and 95th percentiles (whiskers).



Supplementary Figure 1. Endometrial stromal sarcoma-like lesion observed in one *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mouse. **A and B**: Hematoxylin/eosine staining of the affected uterus showing the lesion (red circle indicates the aberrant region); the square indicates the magnified region depicted in figure b **B.** Consecutive sections were stained for SMA (**C**), Estrogen Receptor alpha (**D**) and β-catenin (**E**).

DISCUSSION

The functional role of canonical Wnt/β-catenin signalling in uterine development and homeostasis is to date largely unclear. Recently, Jeong et al. (2009) investigated the effects of conditional activation of Wnt signaling in the uterus by *PgrCre* driven expression of oncogenic β-catenin (*Pgr*$^{Cre/+}$;*Ctnnb1*$^{f(Ex3)/+}$)[10]. The progesterone receptor, however, is widely expressed throughout the uterus from week two after birth onwards, and accordingly a broad spectrum of defects was observed: the *PGR*$^{Cre/+}$;*Ctnnb1*$^{f(Ex3)/+}$ animals showed reduced offspring numbers resulting from a diminished decidual reaction, decreased uterine weight, increased ERα expression in the endometrium, enlarged endometrial glands, endometrial hyperplasia, and increased endometrial proliferation.

In the present study the aim was to investigate the consequences of Wnt/β-catenin activation by Apc deletion in embryonal life for specific adult uterine tissues. In particular, the Amhr2 promoter was employed to drive Cre expression to induce loss of *Apc* function (*Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$) in the myometrium but not in the endometrium. The main difference between the current approach and studies by others on the role of Wnt/β-catenin signaling in the uterus[10-11,24,29] is that here conditional knock-down of *Apc* is used (others use conditional inactivation or activation of β-catenin). The role of APC of course is different from the role of β-catenin: APC is involved in cell adhesion, migration, apoptosis and chromosomal segregation[30]. This is true, however, the main tumor-suppressing function of APC instigates from its role in the Wnt/β-catenin signalling pathway.

Previous studies with the *Amhr2-LacZ* reporter mouse model showed that this promoter is active for a brief period from embryonic day 12.5 to 15.5 in mesenchymal cells surrounding the Müllerian ducts[15,17,31]. Notably, in a related reporter model, *Amhr2Cre*-driven recombination of a Rosa26 reporter (R26R) resulted in β-galactosidase staining of myometrial cells but not in endometrial stromal cells[17,24]. This observation was confirmed in the current study also by assessing somatic recombination of the *Apc15lox* allele in laser-capture microdissected areas of the uterus of *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice (Fig. 1).

A more detailed approach to the determination of Cre-mediate recombination efficiency is represented by β-catenin immunohistochemical staining. Nuclear β-catenin accumulation is often regarded as a hallmark of Wnt/β-catenin signalling activation as the result of loss of Apc function. However, β-catenin IHC analysis of the myometrium

of *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals revealed a majority of cells with cytoplasmatic staining and only very few, sporadic cells with clear-cut nuclear staining. This is consistent with the observed patchy β-galactosidase staining (Fig. 1B). Furthermore, Miclea et al. (2009) and Robanus-Maandag et al. (2010) showed increased intracellular β-catenin staining upon somatic *Apc* deletion with only a minority of cells featuring nuclear accumulation[16,20]. Interestingly these authors did show a clear phenotype resulting from the intestinal Cre-mediated deletion of Apc: tumors in the large intestine[16]. Accordingly, other reports have shown that loss of APC function is necessary but not always sufficient for nuclear β-catenin accumulation [26-27]. In contrast to this, *Pgr*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals did show nuclear β-catenin staining indicating that recombination of Apc can indeed result in nuclear β-catenin staining. There is however a considerable difference between the two models: *Amhr2Cre* is activated only from embryonal day 12.5 to 15.5 while *PgrCre* becomes active and stays active from approximately postnatal day 10 onwards. It is possible that the majority of mesenchymal cells surrounding the embryonal Müllerian duct (where loss of Apc function is established) selectively undergo apoptosis, while more mature myometrial cells in which Apc is knocked down, do not. In fact apoptosis as a result of activation of Wnt/β-catenin signalling has previously been shown for NIH-3T3 fibroblasts[32]. The latter would also be in agreement with the observed loss of myometrial muscle tissue in some of the animals upon *Apc* somatic deletion, described herein (Fig. 2 and 3). Reviewing Müllerian duct sections from embryonal day 14 *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals, however, failed to show increased apoptosis as measured by caspase 3 staining (data not shown). Evaluation of the uterus of *Pgr*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals indicated that it can not be excluded that loss of myometrial muscle fibers develops gradually over a prolonged period of time, or alternatively from pubertal life onwards.

The most prevalent phenotype observed among *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice is undoubtedly represented by the loss and disorganisation of myometrial muscle fibres resulting in a defective myometrium. Myometrial defects were also observed by others by employing comparable models to either induce or inhibit Wnt/β-catenin signalling *in utero*. Arango et al. (2005) used the *Amhr2Cre* model to induce β-catenin depletion and observed profound myometrial defects[29]. In that study, β-catenin depletion in mesenchymal cells surrounding the Müllerian ducts resulted in the appearance of adipocytes replacing myometrial cells. Miller and Sassoon (1997) found that in Wnt7a defective animals, among many other disorders, the myometrial layer became largely disorganized[8]. Tanwar et al. (2009) used a model which mimics more closely our approach in that *Amhr2Cre* was employed to express an oncogenic variant of β-catenin thus

inducing constitutive Wnt activation in the uterus[11]. However, in that study the affected animals developed muscular hyperplasia already at 6 weeks of age, which is substantially different from the current findings in *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice. Tanwar et al. (2009) also observed mesenchymal tumors with characteristics of human leiomyomas and endometrial stromal sarcomas[11]. To investigate whether similar neoplastic lesion could be observed in the *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice, we carefully reviewed all our mice but did not observe any leiomyomas. However, we did observe, in a single mouse, a region reminiscent of an endometrial stromal sarcoma-like lesion (Supplementary Fig. 1). This region was subsequently stained for SMA, ERα and β-catenin. It was observed that ERα and β-catenin expression were both enhanced in this region, which was in agreement with the observations done by Tanwar et al.[11].

Recently, we have extended our analysis on the consequences of loss of Apc function in the uterus by employing *PgrCre* mice (Fig. 3). Myometrial breakdown in *Pgr*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals was to some extent comparable to myometrial defects in *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals. However, *Pgr*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals were also characterized by areas in the myometrium nearing some similarity to human adenomyosis, a condition characterized by the presence of endometrial tissue within the myometrium. Accordingly, Tanwar et al. (2009) occasionally also observed glands and stroma in the muscle of *Amhr2*$^{Cre/+}$;*Ctnnb1*$^{f(Ex3)/+}$ animals[11]. Because in the *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ model the integrity of the myometrium is mainly affected, this seems to indicate that the presence of endometrial tissue into the myometrial layer in the Wnt-activated mice may be the result of a passive process rather than signifying active invasion. Recent investigations by Mehasseb et al (2010) also indicate that invasion of stromal cells from adenomyosis is augmented by the presence of myocytes from affected uteri[33].

In conclusion, we report that *in vivo* inactivation of the endogenous mouse *Apc* gene in mesenchymal cells surrounding the Müllerian ducts results in significant myometrial defects in adult mice. Overall, these results further consolidate the notion that Wnt/β-catenin signalling is important for uterine development and homeostasis.

ACKNOWLEDGMENTS

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3

PROGESTERONE INHIBITION OF WNT/ β-CATENIN SIGNALING IN NORMAL ENDOMETRIUM AND ENDOMETRIAL CANCER

Wang Y, Hanifi-Moghaddam P, Hanekamp EE, Kloosterboer HJ, Franken P, Veldscholte J, van Doorn HC, Ewing PC, Kim JJ, Grootegoed JA, Burger CW, Fodde R and Blok LJ. *Clin Cancer Res*. 2009;15:5784-5793.

ABSTRACT

Wnt signaling regulates the fine balance between stemness and differentiation. Here, the role of Wnt signaling to maintain the balance between estrogen-induced proliferation and progesterone-induced differentiation, during the menstrual cycle, but also during the induction of hyperplasia and carcinogenesis of the endometrium, was investigated. Endometrial gene expression profiles from E_2 and E_2+MPA treated postmenopausal patients were combined with profiles obtained during the menstrual cycle (PubMed; GEO DataSets). Ishikawa cells were transfected with progesterone receptors and Wnt inhibitors DKK1 and FOXO1, measuring Wnt activation. Expression of DKK1 and FOXO1 was inhibited by use of sequence specific short hairpins. Furthermore, patient samples (hormone treated endometria, hyperplasia and endometrial cancer) were stained for Wnt activation using nuclear β-catenin and CD44. In vivo, targets and components of the Wnt signaling pathway (among them DKK1 and FOXO1) are regulated by E_2 and progesterone. In Wnt-activated Ishikawa cells progesterone inhibits Wnt signaling by induction of DKK1 and FOXO1. Furthermore, using siRNA-mediated knockdown of both DKK1 and FOXO1, progesterone inhibition of Wnt signaling was partly circumvented. Subsequently, immunohistochemical analysis of the Wnt target gene CD44 showed that progesterone acted as an inhibitor of Wnt signaling in hyperplasia and in well differentiated endometrial cancer. Progesterone induction of DKK1 and FOXO1 results in inhibition of Wnt signaling in the human endometrium. This Wnt inhibitory effect of progesterone is likely to play a rate-limiting role in the maintenance of endometrial homeostasis and, upon its loss, in tumor onset and progression towards malignancy.

INTRODUCTION

The female sex hormones estradiol and progesterone play rate-limiting roles in the cyclical renewal of the inner layer of the uterus (endometrium). In the first half of the regular menstrual cycle, the proliferation phase, estradiol is required to expand the endometrial layer by inducing cell proliferation. In the second half of the menstrual cycle, the secretory phase, progesterone levels rise, which antagonizes estradiols' proliferative activity by inducing differentiation of epithelial and stromal cells of the endometrium [1]. This, inhibition of estradiol-induced proliferation by progesterone is crucial for the maintenance of homeostasis in the endometrium.

Increased estrogen signaling often underlies endometrial hyperplasia and is a well-established risk factor for endometrial cancer[2]. Because progesterone inhibits estrogen-induced endometrial proliferation, progesterone has been employed in its synthetic form, i.e. medroxyprogesterone acetate (MPA), in palliative treatment of advanced and recurrent endometrial cancer with modest though significant response-rates (15-25%)[3]. Progesterone has also been employed as a primary treatment for endometrial carcinoma confined to the endometrial layer of the uterus, for example in pre-menopausal women determined to preserve fertility. Response-rates in these women can be up to 60%[4-5], indicating that progesterone signaling in well differentiated endometrial cancer is a potent inhibitor of endometrial carcinogenesis.

Wnt/beta-catenin signaling has a central function in the maintenance and control of stem cell compartments where it regulates the fine balance between stemness (Wnt-On) and differentiation (Wnt-Off). This central role in homeostasis of adult stem cell niches is reflected by the frequent association of Wnt/beta-catenin signaling defects with different cancer types (e.g. breast, colon, stomach, liver, ovary, uterine, skin, etc). Central in canonical Wnt signaling is the so called "destruction complex". This complex consists of the scaffold proteins APC (Adenomatosis Polyposis Coli), AXIN1 or AXIN2 (conductin), β-catenin (CTNNB1), and the two kinases CK1 (casein kinase I) and GSK3β (Glycogen Synthase Kinase 3β). In the absence of Wnt ligands formation of the destruction complex induces phosphorylation of β-catenin which results in its degradation. Upon Wnt signaling, the destruction complex is not formed and consequent intracellular β-catenin accumulates and translocates to the nucleus. In the nucleus, β-catenin interacts with members of the TCF/LEF family of transcription factors, thus modulating the expression of a broad spectrum of Wnt downstream target

genes[6]. The latter include genes encoding for proteins with central roles in proliferation and survival, such as cyclin D1 (CCND1) and IGF1 (insuline-like growth factor 1), but also others relevant for migration and invasion (for example CD44). Constitutive activation of the Wnt pathway was first observed in Familial Adenomatous Polyposis where individuals carrying a germline APC mutation developed intestinal polyps upon somatic inactivation of the wild type APC allele. Notably, in the vast majority of sporadic colorectal cancers Wnt signaling is constitutively activated as the result of somatic APC mutations. In the remaining cases, either oncogenic β-catenin mutations or alterations in genes encoding secreted proteins able to inhibit Wnt signaling such as DKK1 (dickkopf homolog 1[7]) are found.

In endometrial cancer, gene mutations leading to constitutive activation of canonical Wnt/β-catenin signaling are an early event[8]. Well differentiated endometrioid adenocarcinomas show nuclear β-catenin accumulation in at least 30% of the cases (31%[9]; 85%[10]). Accordingly, oncogenic β-catenin mutations have been identified in 15-40% of endometrial carcinomas[11-12] whereas loss of heterozygozity of APC was reported in 24% of cases with nuclear β-catenin staining[13]. In addition, the APC A1 promoter was found to be hypermethylated in 47% of endometrial cancers with nuclear β-catenin[13] often in correlation with micro-satellite instability[14]. APC mutation analysis revealed truncating mutations in 10% of all endometrial cancers[15].

In the endometrium, Wnt/β-catenin signaling is involved in a number of developmental processes. For example, it has been shown that Wnt4 is required for Müllerian duct initiation[16], Wnt7A for subsequent differentiation[17] and Wnt5A for posterior outgrowth of the female reproductive track [18]. Furthermore, Wnt/β-catenin signaling signaling has also been implicated in regulation of the normal menstrual cycle[19-20]. Nuclear β-catenin staining, for example, is observed during the proliferative phase of the menstrual cycle[21]. Likewise, Nei et al.[21] showed the absence of nuclear β-catenin staining during the second half (differentiation phase) of the menstrual cycle. Hence, inhibition of Wnt/β-catenin signaling may correlate with the inhibition of estradiol-induced proliferation. Hou et al[22] showed that estrogen-induced endometrial proliferation was effectively inhibited by the Wnt/β-catenin inhibitor SFRP2 and Jeong et al[23] showed that correct β-catenin expression is vital for normal uterine function. As progesterone counteracts the proliferative effects of estradiol during the menstrual cycle, it may do so by inhibiting estradiol-induced Wnt/β-catenin signaling.

Based on the above data, we postulate that progesterone counteracts the proliferative effects of estradiol during the normal menstrual cycle, during hyperplasia

and during early endometrial carcinogenesis by inhibiting Wnt/ β-catenin signaling. According to this hypothesis, sex hormones may modulate Wnt/β-catenin signaling in the normal and aberrant endometrium to maintain the balance between proliferation (Wnt-On) and differentiation (Wnt-Off).

MATERIALS AND METHODS

Patients and patient samples

Postmenopausal hormone treated patients: A description of the inclusion and exclusion criteria, and the histological and molecular findings in the endometrium were documented earlier[1,24]. The study groups were: Control-group (8 subjects, no hormonal treatment)); Estradiol (E_2) group (7 subjects, 2 mg of estradiol administered orally every day, starting 21 days prior to surgery); Estradiol + MedroxyProgesterone Acetate (E_2+MPA) group (6 subjects, 2 mg estradiol + 5 mg MPA administered orally every day, starting 21 days prior to surgery). After treatment, endometrial tissue was dissected-out, RNA isolated and processed, and gene expression was measured using the Affymetrix U133plus2 GeneChips containing 54,614 probe sets, representing approximately 47.000 transcripts (Affymetrix, Santa Clara, CA, USA)

Premenopausal patients during different stages of the menstrual cycle: A description of the inclusion and exclusion criteria, and the histological and molecular findings in the endometrium were documented earlier[25]. The study groups were: PE, proliferative endometrium (4 subjects); ESE , early secretory endometrium (3 subjects); MSE, midsecretory endometrium (8 subjects); LSE, late secretory endometrium (6 subjects). Endometrial tissue was dissected as described above, and isolated RNA was used on the same microarrays as described above (Affymetrix U133plus2 GeneChip).

Endometrial hyperplasia and tumor samples: In total 10 well differentiated tumor samples were stained for PR and CD44. From one representative sample hyperplasia as well as tumor was available. Paraffin embedded samples were obtained from the Erasmus University Medical Center Department of Pathology.

The histopathologic diagnoses of all samples were reviewed by our pathologist, Patricia C Ewing, MD PhD. Descriptions of the samples are given in the Results section and in the Legends to the Figures.

Gene expression data analysis

All raw gene expression data have been posted at the GEO DataSets option in PubMed and are freely available to the scientific community. Raw data of both studies[24-25] were normalized as a group using RMA (Robust Multi-array Analysis) normalization[26]. From the Talbi data[25] we only used gene expression data from well-characterized tissuesamples (Table 1[25] with the exception of samples 455 and 562 that were not made available to the GEO DataSets option in PubMed). SAM (Statistical Analysis of Microarray) was performed in order to identify significant differentially regulated genes between different groups (E_2, E_2+MPA, PE, ESE, MSE, LSE) [27]. The median false discovery rate was set to 1% for these comparisons. Genes with differential expression due to difference in tissue source, rather than hormonal effects, were identified and excluded form subsequent analysis. Cluster analysis was performed as described by Hanifi-Moghaddam et al., 2007[24]. Pathway analyses were conducted using Ingenuity Pathway software (http://www.ingenuity.com).

Cell lines and transfections

Ishikawa cell lines were maintained as described earlier[28]. During the transfection, infection and hormone stimulation experiments the cells were cultured in DMEM/F12 supplemented with 5% dextran-coated charcoal-treated fetal calf serum in the presence of penicillin and streptomycin. Transfection was performed 24h after seeding of the cells (5000 cells per well in a 24-well plate); and 4h after transfection hormones were added to the cells (concentrations are indicated with the figures). At 48h after transfection the cells were harvested to perform reporter assays. The stably PR transfected cell lines used in the shRNA experiments were generated as described earlier[28]; transcient transfections were performed as described earlier[29]. The TOPflash and FOPflash vectors were obtained from Millipore (Upstate, Billerica, MA, USA). DKK1 expression vector (cloned in pcDNA3.1) was obtained from Dr X. He[30], FOXO1 expression vector (cloned in pcDNA3.1) was obtained from Dr J.J. Kim[31] and the dnTCF4 expression vector (cloned in pcDNA3.1) was a gift from Dr H. Clevers[32].

The production of Lentivirus and stable cell line generation was performed as described before[33]. The shRNA specific for hDKK1 (n = 5) and hFOXO1 (n = 6), non-target shRNA control vector, and TurboGFP control vector were obtained from The MISSION™ TRC shRNA libraries from Sigma-Aldrich (St. Louis, MO, USA). The packaging plasmids were provided by the Naldini lab, Vita-Salute San Raffaele Univerity, Milan, Italy.

Immunohistochemistry and Western Blotting

Immunohistochemistry and westernblotting was performed essentially as described before[1,29]. The dilutions of antibody used in Immunohitochemistry were: Rat monoclonal CD44 antibody 1:1000 (BD Pharmingen, San Diego, CA, USA); Rabbit monoclonal ß-catenin antibody 1:4000 (Epitomics, Burlingame, CA, USA); Mouse PR antibody 1:50 (Ab-8 cocktail, Neomarkers, Fremont, CA). The dilutions in Western Blotting were: goat anti-hDKK1 antibody 1:500 (AF1096, R&D Systems, Minneapolis, MN, USA)); rabbit polyclonal anti-hFOXO1 antibody 1:5000 (A300-297A, Bethyl Laboratories, Montgomery, TX, USA); rabbit anti-ß-Tubulin 1:1000 (Ab6046, Abcam, Cambridge, UK).

RESULTS

Progestagens inhibit estrogen signaling during the regular menstrual cycle

Significant Analysis of Microarrays (SAM-analysis) between the endometrial gene expression profiles obtained from untreated postmenopausal women in comparison to women treated for 21 days with E_2 (estradiol) or E_2 + MPA (estradiol + MedroxyProgesterone Acetate) indicated that 5932 probesets (representing approximately 4500 genes) were significantly up- or down-regulated ($p < 0.00015$)

Cluster analysis of these significantly up- or down-regulated genes reveals three distinct dendrogram branches: one cluster encompassed all endometrial gene expression profiles from E_2-treated women, the second contained profiles from untreated controls, and the third profiles from both E_2+MPA-treated women and untreated controls (Fig. 1a). Ten genes were investigated using quantitative real time RT-PCR in a previous study to validate differential expression[24]. In Figure 1b, highly E_2 regulated genes are shown to be counteracted (compensated for) by the addition of MPA. In total, 438 genes were identified as being significantly E_2 regulated i.e. by more than 3-fold up- or down-regulated (Fig 1b, closed bars). For 377 (233 + 144) of these genes, this E_2-regulation was counteracted (compensated for), either in part, or fully, by simultaneous MPA administration (Fig 1b, open bars). These analysis show that treatment with E_2 alone has a profound impact on endometrial gene expression, and that this effect is greatly diminished upon addition of MPA to the E_2 treatment.

We next integrated our profiling results with previously published gene expression data obtained during the main phases of the normal menstrual cycle[25]. SAM analysis revealed a total of 11866 probe sets (representing approximately 9000 genes)

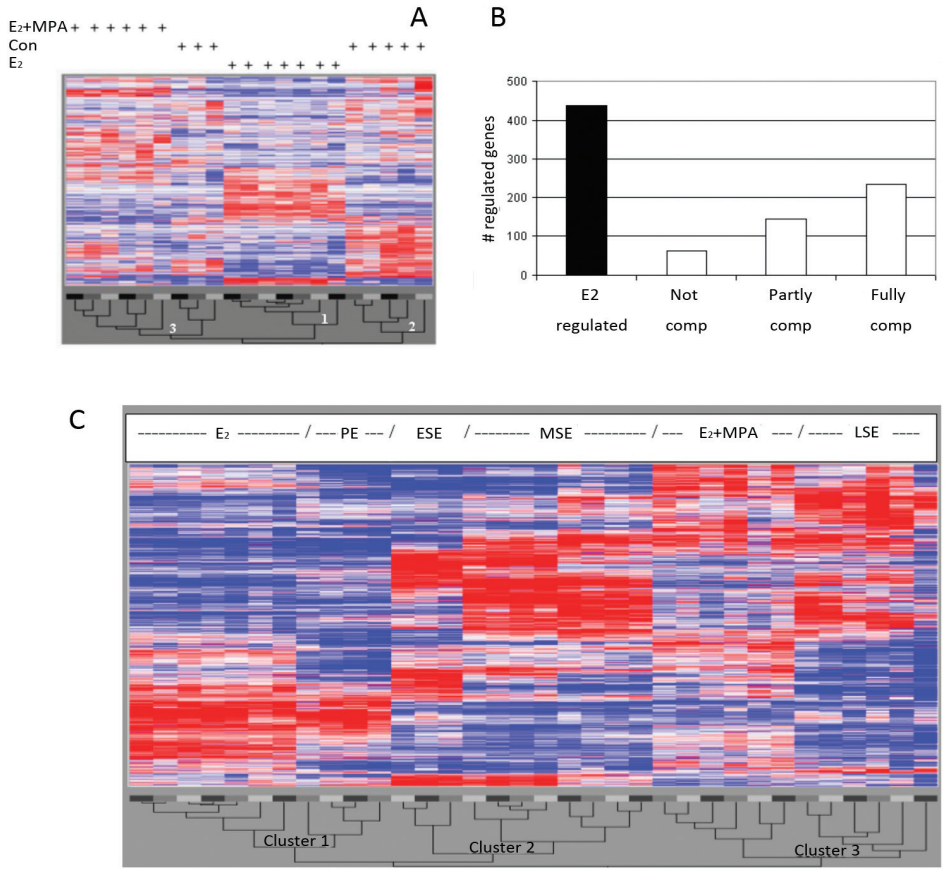

Figure 1. Steroid hormone regulation of endometrial gene expression. A: Cluster analysis of endometrial gene expression profiles obtained from postmenopausal women after no treatment (Con), 21-day treatment with E_2, or E_2 + MPA. Using statistical analysis (SAM), it was established that 5932 probesets (representing approximately 4500 genes) were significantly regulated between the three groups. At the bottom of Figure 1A relatedness is indicated. Red indicates genes with a higher expression relative to the geometrical means, while blue indicates genes with a lower expression relative to the geometrical means. B: Number of genes (# regulated genes) that were regulated at least 3-fold by E_2 treatment (438 genes, closed bar) were also assessed in the E_2 + MPA treatment group (open bars). Not comp: addition of MPA to E_2 did not result in a change in the expression levels of these E_2 regulated genes. Partly comp: addition of MPA to E_2 resulted in a partial compensation (between 1- and 3-fold) of the expression levels of these E_2 regulated genes. Fully comp: addition of MPA to E_2 resulted in a change in the expression of these E_2 regulated genes back to the control levels (less than 1-fold regulated). C: Cluster analyses of endometrial gene expression profiles obtained after 21-day treatment with either E_2 or E_2 + MPA, and during the proliferative endometrium (PE), early secretory endometrium (ESE), mid secretory endometrium (MSE), or late secretory endometrium (LSE) phases. Using statistical analysis (SAM), it was established that 11866 probesets (representing approximately 9000 genes) were significantly regulated between the three groups. At the bottom of the figure, relatedness and Clusters are indicated. Red indicates genes with a higher expression relative to the geometrical means, while blue indicates genes with a lower expression relative to the geometrical means.

significantly regulated between any of the six indicated groups (the E_2 and E_2+MPA treated endometria from the present study, and four different phases of the menstrual cycle; proliferative endometria (PE), early secretory endometria (ESE), mid-secretory endometria (MSE), late secretory endometria (LSE)[25]). These differentially expressed genes were employed for cluster analysis (Fig. 1c). Three clusters were recognized, representing: Cluster 1) the samples from E_2 treated patients and those from women in proliferative phase, Cluster 2) early- and mid-secretory phase specimens, and Cluster 3) the gene expression files from the E_2+MPA treated patients and from women in late secretory phase. These results indicate that estradiol signaling is a very important factor during the proliferative phase of the menstrual cycle (Fig. 1c, Cluster 1). Furthermore, the addition of MPA to the estradiol treatment creates an endometrium with obvious similarities to the late secretory phase of the menstrual cycle (Fig. 1c, Cluster 3).

Progesterone inhibition of Wnt/β-catenin signaling involves both DKK1 and FOXO1

The gene expression data from Figure 1C were subsequently used in a pathway analysis (http://www.ingenuity.com). It was observed that a number of pathways were significantly regulated by one of the treatments or in different phases of the menstrual cycle. For example, when we compared the proliferative endometrium to the early or mid-secretory endometrium, "cancer", "reproductive system disease" and "gastrointestinal disease" were all significantly regulated ($p<0.006$). It is interesting to note that "gastrointestinal disease" and "cancer" both were found to be significantly regulated, which could point to involvement of Wnt/β-catenin signaling. Upon assessing the involvement of "canonical pathways" it was found that "Wnt/β-catenin signaling" was also significantly regulated ($p< 0.003$) in both the early and the mid-secretory endometrium compared to the proliferative endometrium.

Because of our interest in the Wnt/β-catenin signaling pathway we decided to perform a more thorough pathway analysis for either downstream targets or integral parts of the Wnt/β-catenin signaling pathway (http://www.stanford.edu/~rnusse/wntwindow.html[34-35]) (Supplementary Table 1). Upon performing this analysis, a large number of differentially expressed genes (n=228) were recognized. Because our investigations center on the mechanism through which progesterone counteracts the proliferative effects of estradiol during the menstrual cycle and during early endometrial carcinogenesis, we have focused on the progesterone-induced Wnt/β-catenin signal-inhibitor DKK1[36] and the alleged inhibitor FOXO1[37-38] (Fig. 2).

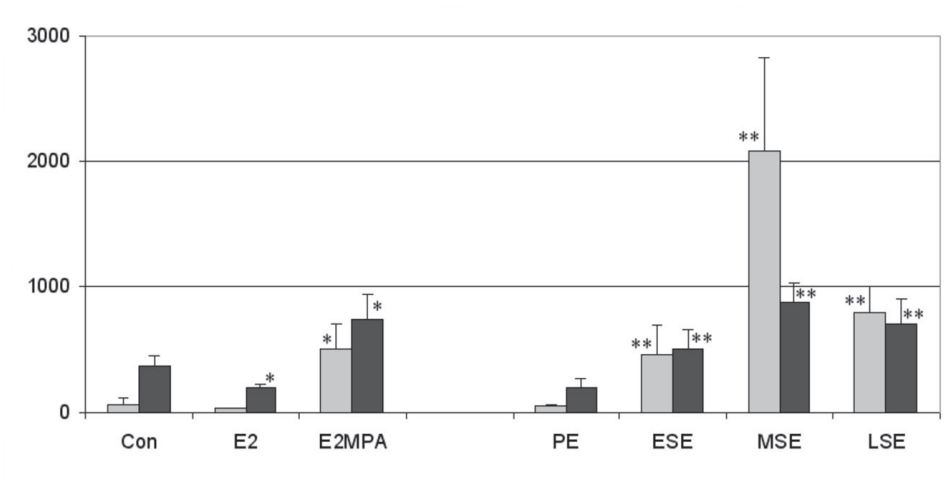

Figure 2. Endometrial expression of DKK1 (black bars) and FOXO1 (gray bars) during steroid hormone treatment (Con, E2 and E2+MPA) and during the menstrual cycle. Microarray data of DKK1 and FOXO1 are plotted. The FOXO1 data represent the average of the data obtained from 4 different probesets (see Supplementary Table 1). On the Y-axis the normalized expression levels are indicated, on the X-axis the different experimental groups are indicated. Con = non treated, E2 = Estradiol treated (21 days), E2 + MPA = Estradiol + MedroxyProgesterone Acetate treated (21 days), PE = proliferative endometrium, ESE = early secretory endometrium, MSE = mid secretory endometrium, LSE = late secretory endometrium (Con, E2 and E2+MPA data are from [24]; PE, ESE, MSE and LSE data are from [25]). Differences between Con and hormone treatments were considered significant (*) when $p < 0.05$. Differences between PE treatment and other stages of the menstrual cycle were considered significant (**) when $p < 0.05$.

In order to investigate the molecular mechanisms underlying progesterone-driven inhibition of Wnt/β-catenin signaling, we employed the well differentiated Ishikawa endometrial cancer cell line[39]. As shown in Figure 3a, TOP/FOPflash reporter assay analysis indicates that Wnt/β-catenin signaling is constitutively active in Ishikawa cells. Furthermore, we could clearly show that Wnt/β-catenin signaling is inhibited upon MPA treatment (Fig. 3a). The concentration of MPA necessary to fully inhibit Wnt/β-catenin signaling is approximately 0.1 nM and this is close to the reported dissociation constant for both progesterone receptor isoforms[40]. Using this ligand concentration (0.1 nM), molecular excess of the antiprogestagen Org-31489 could fully reverse Wnt/β-catenin signaling inhibition. Furthermore, it was shown that both activated progesterone receptors (PRA and PRB) could inhibit Wnt/β-catenin signaling, although PRB did so at lower MPA concentrations (Supplementary Figure 1).

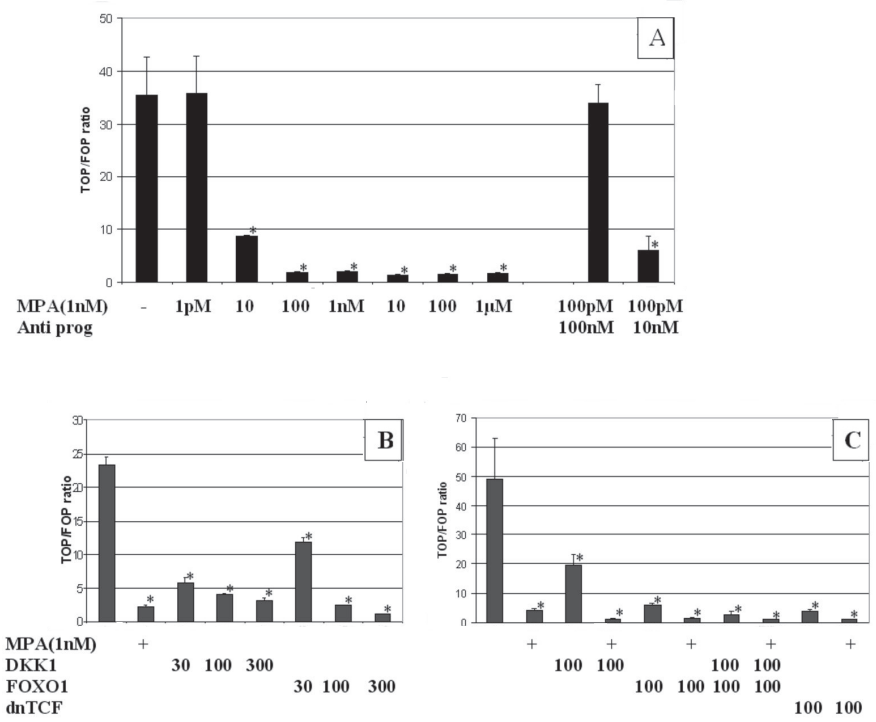

Figure 3. Progesterone, DKK1 and FOXO1 inhibit Wnt/β-catenin signaling in Ishikawa cells. Ishikawa cells were transfected with the TOP-FLASH Wnt/β-catenin reporter construct and several other constructs as indicated in the figure (plasmids are depicted in nanogrammes per well of a 24-well culture plate). Furthermore, the cells were cultured for 48h in the presence or absence of MPA or the antiprogestagen Org-31489 (amounts are indicated in the figure[41]). dnTCF represents a dominant negative inhibitor of Wnt signaling[32]. All experiments are corrected for transfection efficiency by using the tk-renilla vector. The experiments performed under A, B and C have all been repeated at least three times and are independently performed from each other. Differences between non treated and treatments and/or transfections were considered significant (*) when $p < 0.05$.

As shown in Figure 2, the expression of the Wnt/β-catenin signaling inhibitors DKK1 and FOXO1 is elevated during the early secretory phase of the menstrual cycle, peaks during the mid secretory phase and declines during the late secretory phase (Fig. 2). Furthermore, for DKK1 direct progesterone receptor activation of the promoter region was measured (Supplementary Fig. 2). These findings could indicate that progesterone inhibits Wnt signaling by inducing the expression of DKK1 and FOXO1 during the early and mid secretory phase of the menstrual cycle. As shown in Figure 3b, transfection of Ishikawa cells with increasing amounts of DKK1 or FOXO1 expression vector (30ng, 100ng and 300ng per well) results in the progressive inhibition of Wnt/β-catenin

signaling. When cells were transfected with DKK1 or FOXO1 in the presence of MPA (Fig. 3c), or with both DKK1 and FOXO1 together, Wnt/β-catenin signaling inhibition was even more pronounced. Notably, inhibition of Wnt/β-catenin signaling by DKK1 and FOXO1 together was comparable to inhibition by the strong dominant negative expression vector dnTCF1[32].

In order to further substantiate that induction of DKK1 and FOXO1 by progesterone is central to inhibition of Wnt/β-catenin signaling, expression of both DKK1 and FOXO1 was reduced by the use of lentiviral sequence-specific short hairpin RNAs. Five DKK1 and 6 FOXO1 targeting lentivirusses were employed to infect Ishikawa cells. As shown in Figure 4a, partial inhibition of progesterone induction of DKK1 and FOXO1 expression was achieved by infection with combinations of all available specific DKK1 or FOXO1 shRNAs. Based on these results, Ishikawa cells infected with either DKK1- or FOXO1-specific shRNAs were then used to measure progesterone inhibition of Wnt signaling. MPA inhibition of TOP-FLASH Wnt/β-catenin reporter system was partly circumvented by the lentiviral shRNAs directed against DKK1 and FOXO1 (Fig. 4b). Furthermore, prevention of MPA activity was observed when DKK1 and FOXO1 shRNAs were combined. In conclusion, progesterone-induced upregulation of DKK1 and FOXO1 is responsible, in part, for Wnt/β-catenin signaling inhibition in the endometrium.

Progesterone inhibition of Wnt/β-catenin signaling in normal endometrium and endometrial cancer.

It has been reported that at least 30% of early estrogen-associated tumors (Type I endometrioid) display the hallmark of Wnt signaling activation, namely nuclear β-catenin accumulation[9,13]. We performed immunohistochemistry on histological sections from 10 well-differentiated endometrium carcinomas, and three of these showed nuclear β-catenin staining (Fig. 5a, left panel). To confirm that intracellular β-catenin accumulation was accompanied by upregulation of known Wnt/β-catenin downstream targets, CD44 immunohistochemistry analysis was also performed on these three nuclear β-catenin positive tumors[42]. As shown in Figure 5a, CD44 expression coincides very well with nuclear β-catenin accumulation. Moreover, in regions where β-catenin staining is present but not nuclear, CD44 staining is undetectable (Fig. 5a, arrowheads).

In agreement with our hypothesis that estradiol induces Wnt/β-catenin signaling to trigger proliferation of the endometrium, it was observed that expression of the

Wnt target gene CD44 in the endometrium of the seven E_2 treated women was higher than in the six E_2+MPA treated women (Fig. 5b). Furthermore, when CD44 expression was measured during hyperplasia, CD44 expression became markedly reduced upon treatment with progestagens (supplementary Fig. 3).

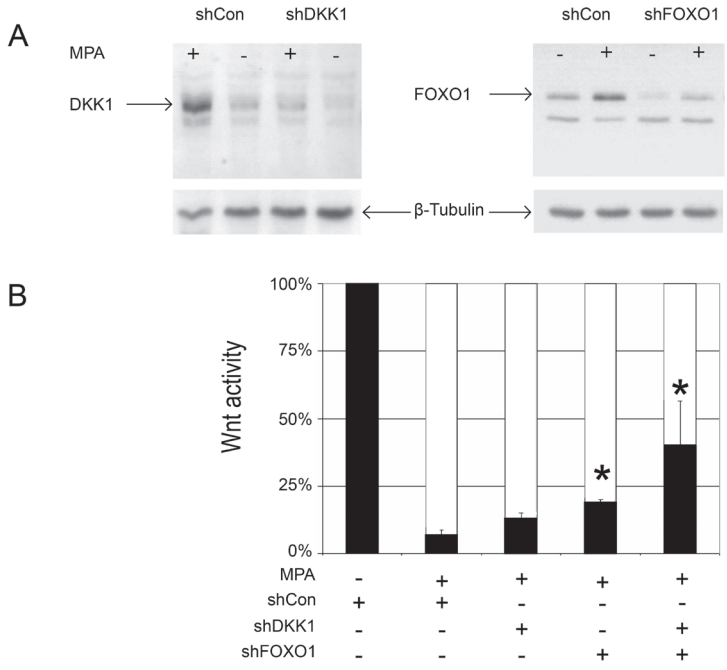

Figure 4. Inhibition of DKK1 and FOXO1 expression partly circumvents MPA inhibition of Wnt/β-catenin signaling. A: Activity of shRNAs directed against DKK1 or FOXO1 were tested in PR expressing Ishikawa cells. B: Ishikawa cells infected with shRNA were transiently transfected with PRA, PRB and the TOP-FLASH Wnt/β-catenin reporter system. The hatched bars indicate MPA inhibition of Wnt/β-catenin reporter activity in the presence of Control (shCon), DKK1 (shDKK1) and/or FOXO1 (shFOXO1) shRNAs. All experiments are corrected for transfection efficiency by using the tk-renilla vector. Differences between MPA treatment and non treated or shRNA treated were considered significant (*) when $p < 0.05$.

This particular patient underwent hysterectomy because of the development of a well differentiated endometrial carcinoma. Immunohistochemical staining for CD44 in this tumor showed a heterogeneous staining pattern. In some fields it was clear that staining for CD44 was positive in areas of carcinoma while adjacent areas of hyperplasia were negative (Fig. 6a). Endometrial tumors often progress to become progestagen-independent and therefore progesterone receptor expression was also assessed by immunohistochemistry and compared with CD44 staining (Fig. 6b). Epithelial CD44 staining was minimal in areas where expression of the progesterone receptor was

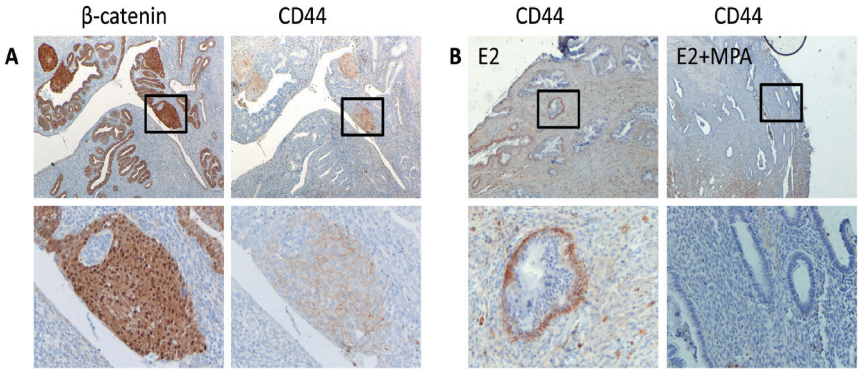

Figure 5. Wnt/β-catenin signaling in different endometrial samples. A: β-catenin and CD44 staining of an area of endometrial hyperplasia in a uterus containing a well-differentaited adenocarcinoma. Arrowheads indicate a region with cytoplasmic β-catenin staining which is devoid of CD44 staining. The higher power shows a squamous morule, where it is recognized that nuclear β-catenin staining is particularly intense [43]. CD44 also stains intensely in this area. B: CD44 staining of the endometrium after 21 days of E_2, or E_2 + MPA treatment[1].

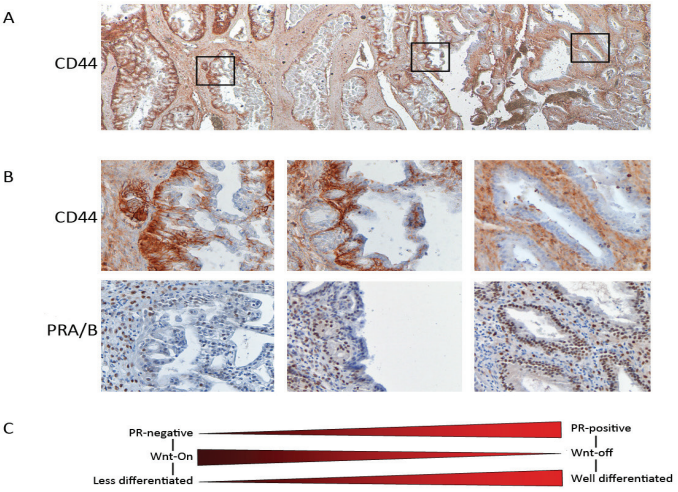

Figure 6. Progesterone regulation of Wnt/β-catenin signaling in endometrial cancer. A: Overview of CD44 staining of endometrial carcinoma during progestagen treatment (approximately 40-fold magnification). B Detail (approximately 200-fold magnification) of staining of endometrial carcinoma for CD44 and progesterone receptors A and B during progestagen treatment. The images on the left are from an area of complex cribriform growth representing adenocarcinoma. The images on the right are from an area where the morphology is still that of hyperplasia. C: synopsis of the findings presented in B: In those regions where progesterone signaling is active (PR-positive), epithelial CD44 staining is reduced and Wnt/β-catenin signaling is inhibited. In those regions that have become progesterone-insensitive (PR-negative), CD44 staining is enhanced and Wnt/β-catenin signaling is no longer inhibited.

positive (Fig 6b, closed arrows). Conversely in regions devoid of progesterone receptor signaling, CD44 levels were clearly enhanced (Fig 6b, open arrows). In order to further substantiate our findings, we reviewed 10 cases of well differentiated endometrial cancer. In all these cases, when the progesterone receptor was present, CD44 levels were low or undetectable, and in those regions of the tumor where the progesterone receptor was lost, CD44 expression was high (supplementary Fig. 4). These data suggest that Wnt/β-catenin signaling can be inhibited by progesterone in estrogen-associated endometrial hyperplasia and cancer, but only in the presence of progesterone receptors (Fig. 5 and 6).

DISCUSSION

Sustained or unopposed estrogen signaling in the endometrium results in hyperplasia[44], which is followed in approximately 25% of cases by tumor formation[2]. Two groups of women are known to be at high-risk for endometrial cancer because of sustained or unopposed estrogen-like activity: those who are significantly overweight[45] (relative risk > 4) and those receiving tamoxifen (a partial oestrogen antagonist) for breast cancer treatment[46] (5 year usage relative risk > 7). It has been estimated that up to 40% of all endometrial cancers may be related to obesity, a figure that is rising as the prevalence of obesity increases.

It has been reported that at least 30% of these early estrogen-associated tumors (Type I endometrioid) display the hallmark of Wnt/β-catenin signaling activation, namely nuclear β-catenin accumulation[9,13], suggesting a cause-effect relationship between sustained estrogen signaling and aberrant Wnt/β-catenin signaling. Accordingly, we found a significant number of targets and components of the Wnt/β-catenin signaling pathway among the genes differentially regulated in the endometrium of E_2 treated women (Supplementary Table 1). This is indicative of the impact of estrogen signaling on this pathway. For example, the Wnt/β-catenin signaling target IGF-1, regarded as one of the most important endometrial growth factors[47], was found to be strongly up-regulated (12-fold) by estradiol and inhibited after the addition of progesterone. In literature there are also a number of reports implicating progesterone and estradiol in regulation of Wnt/β-catenin signaling in the endometrium. Cloke et al.[48] for example, showed that knockdown of the progesterone receptor resulted in activation of Wnt/β-catenin signaling in differentiating human endometrial stromal cells. In agreement

with this, Satterfield[49] could show that progestagens, administered to pregnant sheep induced a transient decline in Wnt/β-catenin signaling activity. Seemingly in contrast to these finding, Rider[50] showed that estrogens activated Wnt/β-catenin signaling in stromal cells, but only after initiation by progestagens. Finally, Katayama[51] showed that administration of an estrogen agonist to immature female rats resulted in down regulation of Wnt7A, and up-regulation of Wnt4 in the uterus, while Catalano[52] showed upregulation of Wnt5A in the endometrium by antiprogestagen treatment in healthy volunteers. These reports and our own observations led us to hypothesize that Wnt/β-catenin signaling is stimulated by estrogens and inhibited by progestagens during the menstrual cycle.

In order to investigate steroid regulation of Wnt signaling during the menstrual cycle, the possible involvement of the progesterone-induced Wnt/β-catenin signaling inhibitors DKK1 and FOXO1 was investigated by use of the well differentiated endometrial cancer cell line Ishikawa. For DKK1 there is good evidence that this protein can bind to the Wnt co-receptors LRP5 and LRP6, thus inhibiting Wnt/β-catenin signaling[53]. For FOXO1 the evidence is scarcer. Essers et al.[54] reported that FOXO1 directly binds to β-catenin and Almelda et al.[37] could show that this binding results in inhibition of Wnt/β-catenin signaling. In the endometrial cancer cell line Ishikawa, where we could show that Wnt/β-catenin signaling was constitutively active, progesterone effectively inhibited Wnt/β-catenin reporter activity (Fig. 3a). Furthermore, upon progesterone treatment there was upregulation of DKK1 and FOXO1 expression (Fig. 4 and[31]), an observation in keeping with the profound up-regulation of the expression of both genes during the secretory phase of the menstrual cycle (Fig. 2 and[20]). Interestingly, Tulac et al.[20], but also Kane et al[55], show progesterone induced DKK1 expression specifically in stromal cells of the endometrium. In analogy to Wnt5A, which is produced by stromal cells and acts on uterine glands[18], it is possible that stroma-produced DKK1 is specifically important to inhibit Wnt/β-catenin signaling in glandular and luminal epithelial cells of the endometrium. The fact that here we are using a well differentiated endometrial cancer cell line of epithelial origin, which shows progesterone induction of DKK1, on the one hand does not represent the physiological situation of the menstrual cycle, but on the other hand does allow us to dissect out the role of progesterone in Wnt/β-catenin signaling regulation.

Upon transfection of Ishikawa cells with vectors encoding either DKK1 or FOXO1, Wnt/β-catenin signaling was clearly inhibited thus indicating that both proteins can serve as Wnt/β-catenin signal inhibitors in response to progesterone. Indeed, inhibition

of Wnt/β-catenin signaling by progesterone treatment was partly circumvented by shRNA-driven down-regulation of DKK1 or FOXO1 expression.

By using the Wnt/β-catenin downstream target CD44 as a marker for Wnt signaling, it was shown that E_2 treatment seems to result in enhanced Wnt/β-catenin signaling. CD44, hyaluronic acid receptor, in this respect is an interesting marker because it has been implicated in progression and metastasis of a number of different cancers[56]. E_2 induced CD44 expression was counteracted (compensated for) by MPA treatment (Fig. 6b). Furthermore, estrogen-induced endometrial hyperplasia[44], also displayed increased CD44 expression which was shown to be inhibited by progestagen treatment (Supplementary Fig. 3). Notably, in an endometrial carcinoma arising during treatment of endometrial hyperplasia with progestagen, areas with enhanced Wnt/β-catenin signaling (CD44 positive) generally corresponded to areas of carcinoma while regions that displayed reduced Wnt/β-catenin signaling (CD44 negative) tended to correspond to areas with the morphology of hyperplasia. Staining the same regions for progesterone receptor expression indicated that the presence of progesterone receptors correlated with a decrease in Wnt/β-catenin signaling whereas its absence coincided with enhanced Wnt/β-catenin signaling. Similar observation were done in other well differentiated endometrial tumors (Supplementary Fig. 4) and several years ago by Hanekamp, who could also show a clear correlation between the absence of PRs and the presence of CD44[57].

In endometrial hyperplasia and cancer we have thus observed that when progesterone receptor signaling is intact, progesterone indeed seems to be able to inhibit Wnt signaling (inhibit CD44 expression). This inhibitory effect of progesterone most likely limits cancer progression, which is observed in 15-25% of patients with metastatic endometrial cancer who respond to progesterone therapy[3,58]. Progesterone treatment, however, only renders a temporary relief and the disease often becomes progesterone-insensitive[58]. Based on the findings of the present study, it seems warranted to consider treatment of endometrial cancer patients whose disease has become progesterone resistant with inhibitors of the Wnt/β-catenin signaling pathway[59] that are acting down-stream of progesterone signaling. Endostatin has been shown to inhibit Wnt/β-catenin signaling through inhibition of LEF1[60]. LEF1 is an important part of the Wnt/β-catenin pathway which we have shown to be up-regulated by estrogens during the proliferative phase of the menstrual cycle and down-regulated by progestagens during the secretory phase of the menstrual cycle[25] (Supplementary Table 1). Endostatin may thus be a potential therapeutic option in progesterone insensitive metastatic endometrial cancer.

In summary, our results provide support for the hypothesis that during the proliferative phase of the menstrual cycle increased estradiol levels induce Wnt/β-catenin signaling to enhance proliferation, while during the secretory phase progesterone levels inhibit Wnt/β-catenin signaling thereby counterbalancing estradiol-induced proliferation and enhancing differentiation. Furthermore, progesterone also seems to be able to inhibit Wnt/β-catenin signaling during endometrial carcinogenesis thus inhibiting the disease. In addition, we provide mechanistic evidence that the Wnt/β-catenin signaling inhibitors DKK1 and FOXO1 are acting down-stream of the progesterone receptor to trigger inhibition of Wnt/β-catenin signaling.

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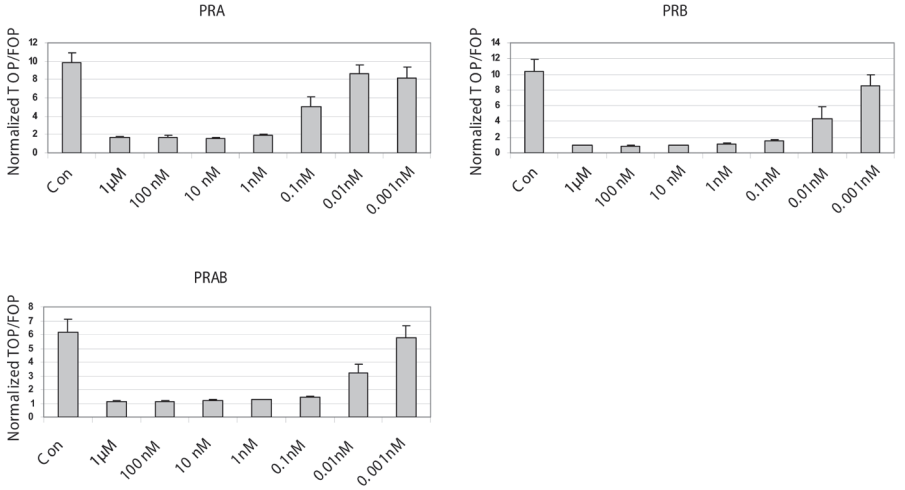
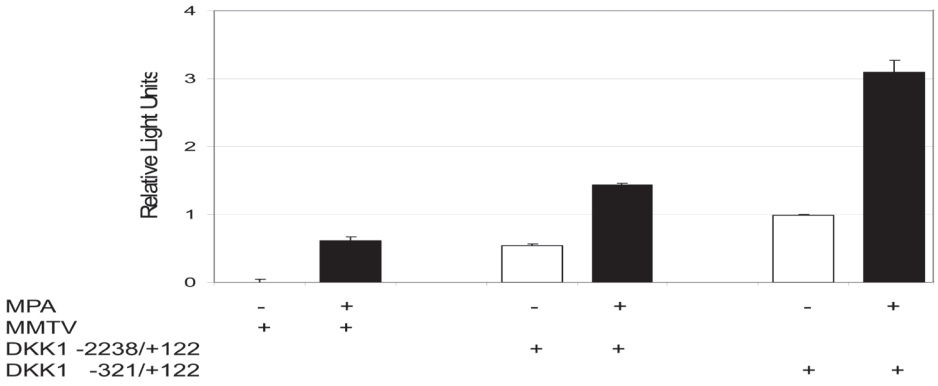
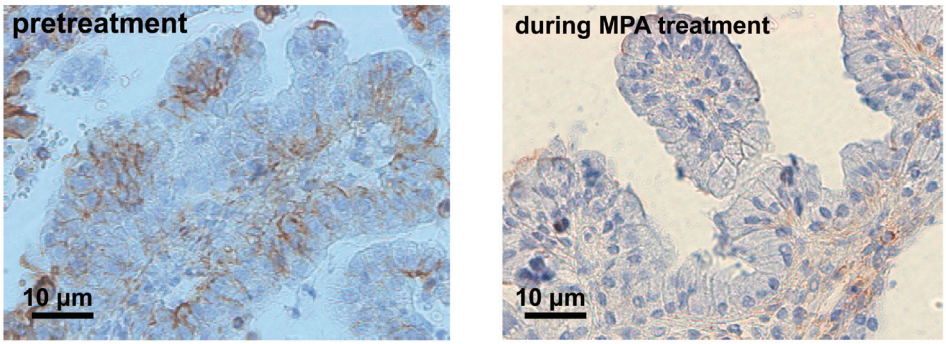


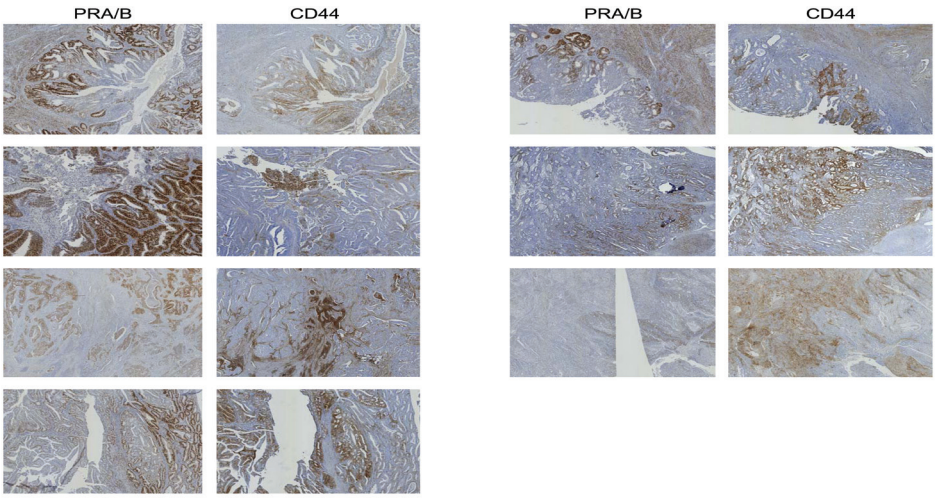
Supplementary Figure 1. Progesterone inhibition of Wnt/β-catenin signaling in Ishikawa cells transfected with PRA, PRB or both progesterone receptors. Ishikawa cells were transfected with the TOP-FLASH Wnt/β-catenin reporter construct and either PRA, PRB orboth progesterone receptors (30 ng/well). Furthermore, the cells were cultured for 48h in the presence or absence of MPA (amounts are indicated in the figure) All figure). experiments are corrected for transfection efficiency by using the tk-renilla vector. The experiments have all been repeated at least three times. The error bars represent the standard deviation of three transfections within one experiment.



Supplementary Figure 2. MPA induction of DKK1 promoter-luciferase reporter constructs. Ishikawa cells were transfected with PRA and PRB, the progesterone inducible construct MMTV-luciferase (MMTV) and two different DKK1-promotorluciferase reporter constructs (DKK1 -2238/+112 and DKK1 -321/+112). The cells were subsequently cultured in the presence or absence of MPA (1nM) for 48h before harvest and measurement of luciferase activity. All experiment are corrected for transfection efficiency by using the tk-renilla vector.



Supplementary Figure 3. CD44 staining of endometrial hyperplasia before (pretreatment) or after (during MPA treatment) progestagen treatment (approximately 200-fold magnification).



Supplementary Figure 4. Progesterone regulation of Wnt/β-catenin signaling in 7 different endometrial cancer samples. Details (approximately 40-fold magnification) of staining of endometrial carcinoma for CD44 (right) and progesterone receptors A and B (left). In those regions where progesterone signaling is active (PRpositive), epithelial CD44 staining is reduced; in those regions that have become progesterone-insensitive (PR-negative), CD44 staining is enhanced.

SUPPLEMENTARY TABLE 1

Endometrial gene expression data from hormone treated postmenopausal women and from women during different stages of the menstrual cycle. The genes are indicated on the right; for most genes, data off more than one probeset were available from the microarray.

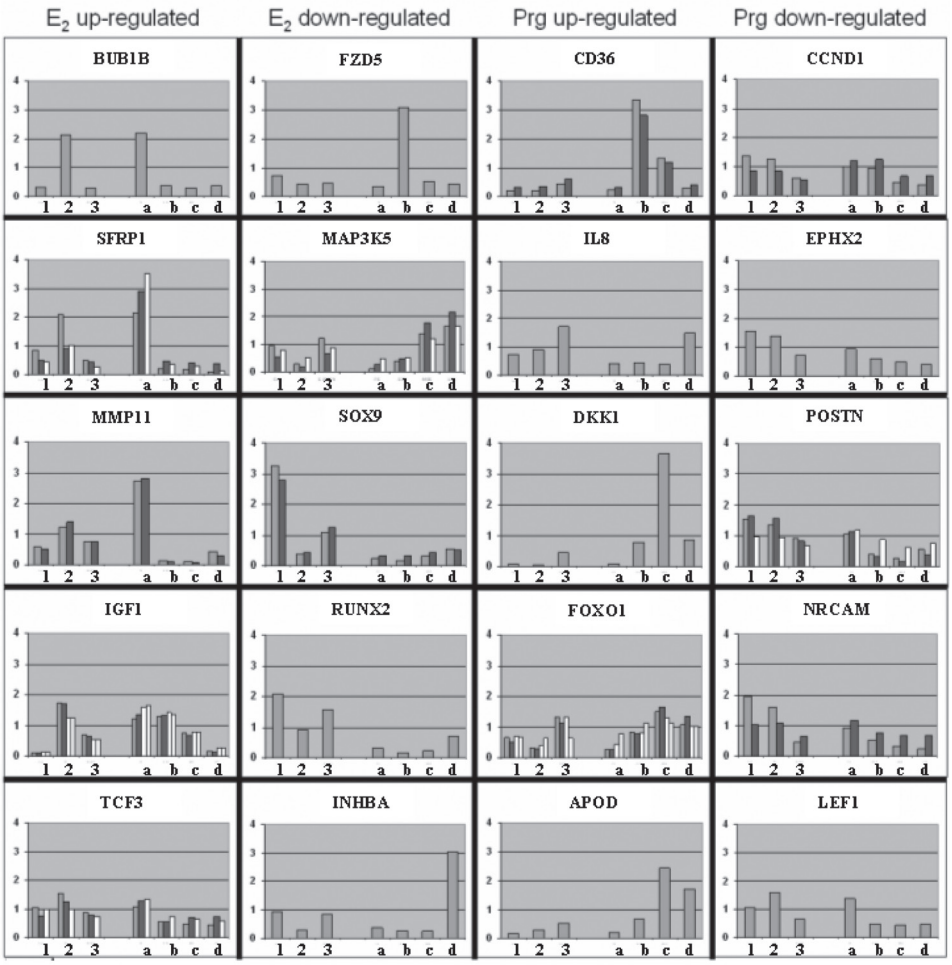
	Con Gene expr value	E2 treated Gene expr value	E2MPA treated Gene expr value	Proliferative endom. Gene expr value	Early Secret Endom. Gene expr value	Mid Secret Endom. Gene expr value	Late Secret Endom. Gene expr value	Gene name	Probe set
1	69.6	54.9	96.3	42.1	47	59	83.3	ABCB1	209993_at
2	679.5	271.2	556.9	142	300.1	263.8	179.4	AKAP12	227530_at
3	168.2	49.7	139	32.4	51.6	49.2	37.1	AKAP12	227529_s_at
4	1058.5	500.6	1079	264.8	368.1	483.5	332.7	AKAP12	210517_s_at
5	141	34.9	92.1	30	33	33.1	30.1	AKAP12	231067_s_at
6	224.1	363.3	289.4	548.8	411.9	443.8	369	AKT1	207163_s_at
7	192.7	88.1	124.3	46.6	66.3	62.9	67.7	AKT3	242876_at
8	568.7	355.7	479.9	182.5	201	208	228	AKT3	212609_s_at
9	334.8	179.1	357.7	174.4	171	194.2	287.7	AKT3	212607_at
10	1315.5	1358.7	1187.3	522	645.6	572.9	525.9	ANXA7	201366_at
11	309	284.1	234.5	89	88.2	91.3	73.9	APC	203525_s_at
12	187.2	316.8	595.2	242.3	741.6	2746.7	1902.7	APOD	201525_at
13	261.6	133.1	228.6	123.5	294.6	166.4	230.4	AXIN1	225557_at
14	150.7	176.9	166.1	311.9	253.4	313.9	297.3	AXIN1	212849_at
15	654.2	515.2	792.9	238.1	284.9	258.7	240	AXIN2	222696_at
16	174.5	187.9	162	287.3	217.9	360.2	237.1	BCR	202315_s_at
17	104.6	109.7	103.6	182.3	151	216.6	153.9	BCR	217223_s_at
18	38.6	121.4	43.9	189.9	60	45.1	53.7	BIRC5	202095_s_at
19	379.3	330.4	556	155.5	637.9	757.6	393.5	BNIP3	201849_at
20	93.1	85.3	144.3	85.5	302.9	297.3	194	BNIP3	201848_s_at
21	325.5	351.8	268.9	239.6	186.3	185.5	157.3	BRD7	221776_s_at
22	360.1	642.5	540	825.9	720.3	1029.1	612.9	BSG	208677_s_at
23	414.2	387.7	372.4	301.7	1505.2	287.2	355.2	BTG3	205548_s_at
24	863	767.1	751.7	563.4	2545.1	564.1	636.3	BTG3	213134_x_at
25	579.6	493.4	455.5	892.6	677	750.9	585.8	BTRC	201730_s_at
26	61	395	53	404.3	71.3	52.9	71.6	BUB1B	203755_at
27	863.4	442.4	1273.8	223.4	494.5	423.5	581.9	CALD1	201617_x_at
28	342.3	226.7	286.6	78.9	111	113.5	188.9	CALD1	205525_at
29	953.6	534.2	1304.6	465.1	703.9	638.5	1076	CALD1	201616_s_at
30	3890.9	2841.5	4547.6	1715.3	2180	2061.7	2572.2	CALD1	212077_at
31	76	61	79.5	55	37.2	62.2	105.9	CALD1	215199_at
32	171.6	152.9	301.1	212.3	276.2	219.7	312.8	CALD1	201615_x_at
33	90	64.9	108.5	120.8	140.6	137.9	201.1	CALD1	243084_at
34	502.2	245.9	261.3	439.6	579.5	441	301.3	CALM2	235190_at
35	171	287.6	265.6	394.6	285.6	383.9	392.5	CALM3	200622_x_at
36	204.4	304.1	227.9	720.6	296.4	474.3	352.2	CALM3	200623_s_at
37	79.9	97.4	81.5	105.1	64	91.3	116.9	CAMK1	204392_at
38	143.2	133.7	119.3	106.8	124.7	117.4	67.9	CAMK2D	231793_s_at
39	107.1	143.6	91.4	61.4	67.5	54	55.1	CAMK2G	239179_at
40	255.8	240.8	484.9	188.1	141.6	142.6	176.8	CAV1	203065_s_at
41	1055.7	1045.8	1872.4	881	486.4	620.3	901	CAV1	212097_at
42	419.1	380.5	188.8	301.5	283.4	142.3	111.5	CCND1	208712_at
43	102.2	100.7	66.9	144.2	151.4	81.3	79.9	CCND1	208711_s_at
44	1953.6	1474.3	1515	783	859.9	911.1	799.5	CCNG1	208796_s_at
45	30.3	30.4	62.6	32.8	484.3	193.3	40.3	CD36	209555_s_at
46	30	30.6	54.6	30	258	108	37.4	CD36	206488_s_at
47	107.2	138.1	117.5	61.8	129	377.5	218.2	CD44	217523_at
48	1218.2	1732.1	1957.1	721	1367	2707.3	1646.5	CD44	212063_at
49	119.8	174.7	202.5	140.3	270.8	641.2	465.8	CD44	212014_x_at
50	336.8	454.6	542.4	269.7	448.9	942.2	605.6	CD44	204489_s_at
51	150.5	211.9	270.7	189.7	304	736.8	473.4	CD44	209835_x_at
52	128.9	190.1	234.8	139.8	218.2	436.6	324.8	CD44	1557905_s_at
53	118.4	166.1	211.5	128.6	181.9	374.2	283.3	CD44	204490_s_at
54	99.2	136.5	146.6	136.7	200.1	406.7	314.9	CD44	210916_s_at
55	32.6	44.8	34.1	39.4	47.6	82.5	69.1	CD44	1565868_at
56	39.5	35.3	37.4	46.2	50.3	70.4	74.6	CD44	216056_at
57	169.5	138	107	93.2	208.3	87.9	75	CDC14A	243640_x_at
58	234.8	120.4	145.9	73.1	320.2	138.1	84.7	CDC14B	221556_at
59	130.4	73.9	87.3	54.3	135.1	73.1	55	CDC14B	208022_s_at
60	1363	1246.4	990.2	942.5	781.8	2331.5	1086.9	CDH1	201131_s_at
61	49.4	43	54.3	79.1	68.8	204.8	87.2	CDH1	201130_s_at
62	491.3	626.2	280.5	519.4	131.5	115.7	214.6	CDH2	203440_at
63	263.2	137.2	377.9	158.2	91.6	195.9	226.2	CDH3	203256_at
64	36.2	169.5	39.3	198	63.1	47.8	67.4	CDKN3	209714_s_at
65	32.3	150.8	37.8	199.7	60.1	45.5	65.2	CDKN3	1555758_a_at

66	153.6	57.9	105.2	49.2	62.5	149.9	105.9	CLDN1	222549_at
67	283.2	184.3	584.4	203.6	288	103.2	199.3	CNN1	203951_at
68	911.8	1342.9	1416.3	630.5	373.3	350.9	323.6	CNN3	201445_at
69	3121.8	2413.8	2001.5	1381.4	737.7	1018.6	970.3	CNN3	228297_at
70	362.4	2055.7	985.9	1532.3	865.1	868.5	587.4	COL1A1	202311_s_at
71	1878.5	11420.8	5735.8	14327.7	8494.4	8234.8	6262.8	COL1A1	202310_s_at
72	322.6	1177.9	575.6	5537.6	1992.2	1948.3	1440.7	COL1A1	217430_x_at
73	327.8	732.4	388.1	591.5	297.3	214.4	392.8	COL7A1	204136_at
74	252.3	553.8	331.6	1015.7	405.7	345.1	573.8	COL7A1	217312_s_at
75	1156.3	1014.2	918	414.6	488.9	436.4	411.5	CREBBP	202160_at
76	682	516.6	459	116.7	210.1	190	212.8	CSNK1A1	242467_at
77	338.8	252.2	274.5	78.1	164.5	129.7	146.9	CSNK1A1	240221_at
78	417.4	300.3	287.2	104.7	117.3	131.5	134.4	CSNK1A1	208866_at
79	93.7	65.9	78.3	63.7	123.5	111	119.2	CSNK1A1	1556006_s_at
80	144.7	174.8	191.4	248.7	259.2	377.8	288.2	CSNK1D	207945_s_at
81	534.1	419.6	433.8	199.1	238.8	230.2	208.9	CSNK1G3	227767_at
82	1343.3	1684.3	2055.8	875.7	286.5	1593.2	1259.3	CSPG2	204619_s_at
83	1870.1	1821.5	3841.8	1923.7	419.1	3324.3	2253.8	CSPG2	215646_s_at
84	766.9	659.7	1545.1	1406.2	311.9	2187	1564	CSPG2	211571_s_at
85	3226.6	3565.4	5080.2	4692	1184.7	5937	4314.7	CSPG2	204620_s_at
86	3974.8	4367.6	5897.1	6089.2	1476.8	7344	5273.5	CSPG2	221731_x_at
87	552.1	346.4	377.3	147	215.4	185.6	263.1	CTNND1	223679_at
88	150.4	128.3	164.3	66.6	83.9	88.1	82.6	CTNND1	1554411_at
89	870.7	500	540.1	366.9	422.3	370.5	467.6	CTNND1	242558_at
90	313	227.6	184.8	230.2	333	283.7	293.9	CTNND1	238884_at
91	295.4	297.4	326	136.9	148.3	146.8	160.6	CUL2	203079_s_at
92	3710.7	3464.3	2785.2	404.2	811.1	587.8	2316.3	CYR61	201289_at
93	2543.9	2280.9	1810	415.7	539.2	441.9	1783.3	CYR61	210764_s_at
94	238.2	254.9	244.9	85.8	38.3	81.3	374.3	DACT1	219179_at
95	50.9	34.8	243.7	48.2	421.2	1976	468	DKK1	204602_at
96	1086.5	1243.3	842	639.1	195.1	86.4	99.9	DKK3	214247_s_at
97	185.4	234.2	147.8	149.9	52.9	35.2	38.2	DKK3	202196_s_at
98	987.9	1181	593	833	119.7	247.8	172.9	DLL1	209031_at
99	66.5	122.1	73.9	159.9	130.2	144.5	113.1	DMPK	37996_s_at
100	2343.4	1727.6	3283.2	1333.3	1761.6	2098.5	1723.1	DSTN	201021_s_at
101	5723.2	4138.9	5799.2	676	3715.2	3355.4	5664.6	DUSP1	201041_s_at
102	303.2	176	314.1	83.9	183.5	193	308.3	DUSP1	201044_x_at
103	141.9	122.3	155.2	93.9	91.6	111.1	155.9	DUSP10	221563_at
104	68.4	62.8	78.9	61.5	63.2	67.9	110.2	DUSP10	215501_s_at
105	165	223.1	223.4	204.3	442.5	264.1	245.9	DUSP14	203367_at
106	442.8	284.7	294	226.4	176.2	210.4	218.1	DUSP16	224832_at
107	363.5	319.4	191.3	243.9	230	263.3	160.9	DUSP2	204794_at
108	118.1	53.2	63.6	39	41.9	45.5	54.9	DUSP4	204014_at
109	115.3	84.4	101	75.8	123.1	135.8	128.9	DUSP4	204015_s_at
110	344.9	219	272.8	143.8	98.7	185.9	319.3	DUSP5	209457_at
111	1301.2	1074.8	818.6	344.6	273.6	448.5	570.7	DUSP6	208891_at
112	873.7	687.8	499.9	248.5	257.5	372.8	472.5	DUSP6	208892_s_at
113	526.6	359.6	340.8	284	227	310.6	366.1	DUSP6	208893_s_at
114	166.7	187.3	123.1	133.9	123.6	128.2	101.2	DUSP8	206374_at
115	809.1	1265.3	937.2	671.7	397.5	402.9	577	EFNB2	202668_at
116	164.7	277.3	184.8	413.1	181.1	205.6	231.2	EFNB2	202669_s_at
117	277.3	420.8	233.9	362.8	193.8	178.4	152	EFNB3	205031_at
118	333.6	414.1	480.9	336.9	405.4	361	546.3	EGFR	1565483_at
119	520.2	368.3	323.9	274.1	251.7	194.2	194.2	EGFR	232541_at
120	283.8	336.4	378.7	326.1	370	315.4	456.1	EGFR	1565484_x_at
121	100.1	101.5	114.9	211.6	165.2	161.4	194.8	EGFR	211607_x_at
122	101	108.8	114.8	213.3	169.3	164.4	181.4	EGFR	210984_x_at
123	886.2	863.9	817	1139.5	677.3	550.8	512.5	EGFR	201983_s_at
124	222.6	274	174.4	69.2	81.5	68.3	59.6	EIF4E	201436_at
125	471.3	610	476.1	219.5	244.7	215.8	189.4	EIF4E	201437_s_at
126	42.5	64.7	51.5	70.7	67.3	115.3	96.9	EN2	207060_at
127	2934.8	1866.2	2354.3	539.1	394.7	464	645.9	ENPP2	209392_at
128	1231.4	664	1034	343.1	233	263.1	344.4	ENPP2	210839_s_at
129	339.3	524.4	342.1	119.2	179.9	174	152.5	EP300	202221_s_at
130	152.7	217.9	152	92.1	104.9	107.6	88.3	EP300	213579_s_at
131	141.2	125.1	63.1	85.1	55.3	45.4	36.5	EPHX2	209368_at
132	32.2	30	45.5	30	30.3	30	78.5	FCGR3A	204006_s_at
133	71.8	57.3	126.6	54.1	68.1	60	132.2	FCGR3B	204007_at
134	291	90.7	207.7	98.2	85.6	159.8	202.5	FGF18	231382_at
135	137.5	64.3	93.3	57	51.2	72.9	77.8	FGF18	211029_x_at
136	122.1	52.8	84.9	57.6	39.3	59.3	71.4	FGF18	206987_x_at
137	1125.6	381.4	362	267.6	270	163.7	73.2	FGF9	206404_at
138	642.9	675	621.8	921.5	71.3	95.9	218.2	FJX1	219522_at
139	47	65.1	58.6	58.2	52.9	61	101.7	FKBP1A	210187_at
140	183.2	350.5	335.2	389.9	294	388.1	393.3	FKBP1A	210186_s_at
141	343.4	648	615	887.2	617.3	914.8	929.8	FKBP1A	214119_s_at
142	735.3	1182	1064	1497.2	947.8	1334.1	1507.6	FKBP1A	200709_at

143	910.5	2805.4	2369.8	2233.2	3975.8	1995.3	1495.5	FN1	212464_s_at
144	1094.4	3389.7	2781.9	2628.9	4595.4	2290.3	1796.5	FN1	211719_x_at
145	982.2	2976.9	2473.9	2614.6	4640.5	2247.7	1774.1	FN1	216442_x_at
146	1012.2	3066.5	2498.9	2726.3	4561.9	2289.5	1791	FN1	210495_x_at
147	812	394.5	1635.6	336.3	1033.2	1859.4	1329.4	FOXO1	202724_s_at
148	320.6	173.7	705.2	169.5	488	1019.1	837.8	FOXO1	232882_at
149	218.5	131.2	417.8	132.4	258.3	408.5	314.6	FOXO1	202723_s_at
150	94.2	90	92	112.7	159.8	161.3	146.4	FOXO1	239728_at
151	165.7	88.8	57.5	51.6	38.9	32.9	46	FRZB	203697_at
152	387.5	490.1	468.3	242.5	462.2	200.1	144.7	FZD1	204451_at
153	36.8	37.6	42.2	84.4	152.2	69.7	52.6	FZD1	204452_s_at
154	694.2	267.2	551	181.9	69.9	88.6	231.6	FZD10	219764_at
155	196.3	281.8	196	245	109.1	84.8	111	FZD2	210220_at
156	117.3	100.5	117.4	54.3	47.1	41.2	44.8	FZD3	219683_at
157	259.8	160.6	171.7	131	1110.8	184.4	153.9	FZD5	221245_s_at
158	507.9	416	379.7	217.9	414	386.3	158.1	FZD6	203987_at
159	112.6	82.2	43.8	31.4	39.7	32.7	33.3	FZD7	203706_s_at
160	50.6	39	30.6	32.6	32.5	30.9	31.2	FZD7	203705_s_at
161	54.2	50.1	58.6	61.1	51.8	341.1	93.9	GAST	208138_at
162	430.8	504.3	361.6	510.7	389.9	239.9	188.4	GATA2	209710_at
163	6118.4	7465.6	6927.8	5125.6	1107.8	3476.4	4520	GJA1	201667_at
164	36.9	84	30.6	50.3	203	31.5	34.7	GJB6	231771_at
165	114.6	30.1	42.9	30.5	30	30	59.8	GREM1	218469_at
166	59.9	30.4	38.3	30	30	30	57.8	GREM1	218468_s_at
167	32.8	52.9	34.1	70.4	83.4	37	41.6	GREM2	235504_at
168	32.4	59.3	30	101.9	195.5	31.4	35	GREM2	220794_at
169	123.7	155.8	120.6	242.9	201	211.6	206.6	GSK3B	209945_s_at
170	53.2	69.8	80.3	118	73.9	88.4	50.2	GSTT2	205439_at
171	123.9	84.8	71	65.5	74.9	60.8	51	GTF2I	232710_at
172	526.3	577.5	468.8	279.7	263.4	230.6	186	HAT1	203138_at
173	664.2	582.8	793.9	896	987.5	856	779.2	HSP90B1	200598_s_at
174	141.5	83.7	145.7	263.7	320.2	235	227	HSP90B1	216450_x_at
175	372.3	308.1	257.4	56.1	129.8	112.8	161	ID2	213931_at
176	427.8	6031.6	2422.1	4187.8	4519.5	2608.9	605.3	IGF1	209541_at
177	229.5	3210.9	1218.9	2576.3	2506.6	1279.1	271.5	IGF1	209540_at
178	157.8	1419.3	635.2	1817.6	1624.2	873.2	267.2	IGF1	211577_s_at
179	171.7	1610.3	713.7	2124.7	1768	997.9	337	IGF1	209542_x_at
180	2239.5	1012.5	5044.1	2901.1	1416.8	2521.5	3333.4	IGF2	202409_at
181	148.1	103.4	308.8	274.9	108.7	262.8	367.5	IGF2	210881_s_at
182	167.7	102.4	382.8	303.9	106.9	277.7	387	IGF2	202410_x_at
183	361.9	2672.5	1095.2	2044.7	1969.5	1379.3	348.2	IGFBP5	211959_at
184	56	77.2	69.1	135.3	130.3	98	64.5	IGFBP5	203425_s_at
185	36.6	59.8	51.8	106.3	77.5	77.8	59.3	IGFBP5	1555997_s_at
186	30.1	32.1	34.9	73	63.2	50.1	35.7	IGFBP5	203424_s_at
187	44	243.1	111.3	323.6	260.5	189.1	80.4	IGFBP5	211958_at
188	174.3	69.4	161.2	45.9	45.1	62.9	85.7	IL6	205207_at
189	81.7	100.4	192.2	44.5	47.9	42.9	167.8	IL8	202859_x_at
190	115.9	36.8	106.3	48.8	30.9	31.4	380.1	INHBA	210511_s_at
191	145.5	136	499.1	46.4	124.1	1396.1	442.6	IRX3	229638_at
192	833.8	1269.4	1259	2099.3	781.1	850.1	1175.2	ISLR	207191_s_at
193	1269	1264.1	936.2	369.5	537.4	548.9	639.2	JAG1	216268_s_at
194	1281.6	1338	990.3	542	712.6	805.6	839.7	JAG1	209099_x_at
195	108.2	115.9	110	76.2	131.7	129.2	129.3	JAG1	209098_s_at
196	77.5	57.6	53.9	49.6	79.8	69.2	85	JAG1	231183_s_at
197	2218.9	1815	1193	184.4	650.8	467.9	453.8	JUN	201466_s_at
198	2359.7	2055	1574.9	606.3	1768.3	1328.2	1526.2	JUN	201464_x_at
199	358.3	229.1	209.7	103.6	205	167.2	206.7	JUN	213281_at
200	341.9	263	211.7	118.7	193.1	167.5	180.8	JUN	201465_s_at
201	166.5	997.3	184.9	1144.3	242.9	144.5	202.2	KIAA0101	202503_s_at
202	877	549.3	897.2	174.8	737.2	811.2	639.4	KLF4	221841_s_at
203	123.7	76.7	141.5	78.6	217.3	234	188.2	KLF4	220266_s_at
204	496.4	183.3	387.8	47.6	339.5	421.2	176.9	KLF5	209211_at
205	166.4	90.9	150	119.5	319.2	438.3	197.8	KLF5	209212_s_at
206	778.7	1172	470.6	1008	353.1	316	337.2	LEF1	221558_s_at
207	2340.3	435.1	764.4	241.8	211.9	371.2	49.4	LGR5	213880_at
208	405.8	90.9	160.2	132.9	99.2	151.1	73.9	LGR5	210393_at
209	836	650.6	588.5	351.8	311.2	264	195.7	LRP6	225745_at
210	131.2	124.1	101.3	65.2	80.3	98.9	63.9	LRRFIP2	239557_at
211	563.8	431.8	665.1	323.5	345.9	520.8	733.2	MAP3K4	216199_s_at
212	564.5	437.3	729.3	371	407	602.5	829.7	MAP3K4	204089_x_at
213	744.3	232.5	960.4	96.1	304.1	1077.1	1276.6	MAP3K5	203837_at
214	218.9	71.6	269	107.2	197.7	730.2	895.1	MAP3K5	203836_s_at
215	48.6	32.4	55.2	30.7	32	76.2	103.1	MAP3K5	242461_at
216	51.1	84.1	74.6	104.5	96.2	137.8	65.7	MAP3K6	219278_at
217	179.1	83.1	174.7	30	36.9	72.6	104.3	MAP3K8	205027_s_at
218	141.3	86.4	136.1	34.5	61	97	139.2	MAP3K8	235421_at
219	67.1	71.6	95.6	101.9	118	115	118.2	MAPK1	208351_s_at
220	77.7	154.2	112.8	165.8	184.3	235.5	215	MAPK13	210058_at
221	77.2	126.9	83.5	132	132	181.3	164	MAPK13	210059_s_at
222	67.7	40.5	46.3	36.6	40.5	53.3	50.3	MAPK14	232876_at
223	70	58.8	95.4	79.8	93.3	142	150.8	MAPK14	210449_x_at
224	81.2	74.5	102.8	93.2	109.4	165.8	162.4	MAPK14	211561_x_at

225	708.9	664.9	933.8	624	808.5	1491.9	1468.2	MAPK6	207121_s_at
226	113.1	133.7	139.7	202	152.2	200.8	271.2	MAPK7	35617_at
227	126.2	150.1	156	242.7	181.8	218.7	286.8	MAPK7	207292_s_at
228	246.3	171.3	168.2	131	134.8	138.1	157.7	MAPK8	243280_at
229	312.5	384.8	302.8	141	142.9	168.7	131	MARK3	202569_s_at
230	73.6	73	53.5	30	30	30.1	30.1	MARK3	232537_x_at
231	746.5	665.7	963.3	425	287.8	1537.7	942.3	MET	203510_at
232	30.1	30.3	32.5	39.2	34.2	82	67	MET	213816_s_at
233	130.3	108.8	169.6	203.4	138.3	389.6	267.9	MET	211599_x_at
234	122.5	102.8	144.5	187.9	144.3	308.2	240.1	MET	213807_x_at
235	1389.3	1084.8	1818.5	614.4	554.3	1304.7	1166.1	MITF	226066_at
236	796.4	665.8	1144.3	520.1	526.8	1040.8	917.9	MITF	207233_s_at
237	125.2	104.6	155.3	115.7	120.2	254.9	243.5	MITF	240555_at
238	30.9	30	311.9	32.9	30	46.5	2505.1	MMP10	205680_at
239	1218.6	2532.7	1558.4	5618.5	322.7	235.8	869.4	MMP11	203878_s_at
240	377.7	1000.9	545	2010.1	76.5	69.5	215.7	MMP11	203876_s_at
241	1609.1	1745	3513.8	2030.3	1429.7	1670.3	4295.6	MMP2	201069_at
242	46.8	604.4	112.8	1463.2	5032	456.1	54.6	MMP26	220541_at
243	42.6	42.8	39.5	65.7	92.3	70.9	66.5	MMP7	235009_at
244	773.9	909	936.9	552.2	589.9	1131.1	422.8	MYC	202431_s_at
245	205.3	130.3	145	91.3	113	120.6	131.8	MYST3	242480_at
246	728.9	693.7	543.8	373.3	340.5	230.5	254.4	MYST4	212462_at
247	75.4	49.6	43.3	49.4	60.6	48.9	36.6	NANOG	220184_at
248	347.1	290.1	273.5	175.5	399.6	169.8	156.9	NLK	222589_at
249	101.9	74.1	70.9	42.4	56	38.4	45.9	NLK	1556568_a_at
250	389.6	320	92.4	187.8	103.2	63.2	47.4	NRCAM	204105_s_at
251	67.4	71.3	42.8	78.3	51	45.2	44.7	NRCAM	243240_at
252	273.1	175.2	170.4	78.9	198	107.9	89	PCAF	203845_at
253	63.4	59.1	44.9	43.8	133.1	134.5	45.1	PHLPP	212719_at
254	35.9	43	39.7	63.8	68.3	64.7	78.4	PHLPPL	214944_at
255	104.1	121.8	103.5	197.1	129.5	209.9	177.6	PKN1	202161_at
256	678.4	483.9	497.1	204.3	283.9	319	256.3	PKN2	212628_at
257	474.2	314.8	362.3	179.2	311.2	276	303.6	PKN2	212629_s_at
258	622.5	641.7	265.7	476.9	841.8	315.2	161.5	PLCB1	213222_at
259	152.9	117.6	67.3	97.7	106.4	69.4	64.3	PLCB1	242494_at
260	452.3	352.7	184	395.6	382.9	218.1	176.3	PLCB1	244726_at
261	70.9	64.9	77.8	150.6	178.4	126.2	115.7	PLCB1	215687_x_at
262	134	211.4	140.7	99	362.5	354.5	124.9	PLCB4	203895_at
263	37.5	44.9	51	54.4	156.9	161.9	53.1	PLCB4	203896_s_at
264	36.8	36.4	41.3	56.9	90.6	86.9	64.5	PLCB4	240728_at
265	64.9	80.1	86.7	98.8	78.9	151.8	133.9	PLCD3	1552476_s_at
266	119.5	242.7	147.5	417	204.7	207.3	159.5	PORCN	219483_s_at
267	196.6	173.2	114.7	133.9	51.1	32.6	69.8	POSTN	1555778_a_at
268	745.6	710.4	376.7	517.9	151	61.2	168.5	POSTN	210809_s_at
269	78.8	76	55	95.6	72.7	49.9	62.4	POSTN	228481_at
270	356	538.4	474.9	756.5	707.8	821	739.7	PP4C	208932_at
271	105	126.5	127.7	55.2	101.9	80.9	81.2	PPM1A	231370_at
272	90.9	114.8	78	36.8	42.9	39.3	36	PPM1A	229027_at
273	438	353.3	445.6	303.8	753	739.3	442.9	PPM1B	209296_at
274	284.5	202.3	244.7	61.7	111.4	115.1	106.4	PPM1D	204566_at
275	193.6	267.3	216.6	404.8	387.3	311.3	258.9	PPM1G	200913_at
276	499.7	442	528	123	340.2	321.2	194.7	PPMB1	213225_at
277	1610.9	1550.8	2169.6	355.8	740.4	832.3	456.5	PPP1CB	201407_s_at
278	1594.5	1389.7	2360.3	510.5	1132.7	1133.5	702.9	PPP1CB	201409_s_at
279	920.2	776.7	1274	313	597.9	567.1	324.3	PPP1CB	201408_at
280	2441	2163.2	1991.8	1548	1077	979.7	835	PPP1CC	200726_at
281	398.8	601.7	468.5	999	700.8	795.2	596.1	PPP2R1A	200695_at
282	78.7	85.6	95	63.4	116.1	194.8	107.5	PPP2R1B	222351_at
283	69.2	60.8	87.8	75.7	139.8	173.9	92.6	PPP2R1B	202886_s_at
284	330.3	435.6	453.1	329.5	236	215.5	336.6	PPP2R3A	209633_at
285	200.6	287.9	215.6	470.4	322.3	330.7	297.9	PPP2R4	208874_x_at
286	169.1	241.2	170.4	420.7	283.2	291.2	257.9	PPP2R4	206452_x_at
287	170.6	233.1	170.1	424.1	261	296.2	258.6	PPP2R4	216105_x_at
288	501.3	455.2	490.8	201.7	424.9	299.1	275.1	PPP3CA	202429_s_at
289	330.2	313.6	299.7	177.9	136.5	147.8	177	PPP3CB	209817_at
290	73.1	71.2	93.5	57.8	94.6	110.2	112.5	PPP3CC	207000_s_at
291	279.7	245	309.4	65.1	101.1	135.2	115	PRKAA1	225984_at
292	185.7	149	266.4	113.4	155.2	143.6	164	PRKACB	202742_s_at
293	596.1	404.3	653.9	228.7	191.4	226.4	251.4	PRKCA	213093_at
294	87.8	57.3	53.9	47.3	44.8	40.9	33.6	PRKCE	216766_at
295	223.3	170.5	242.6	123.8	182.7	202.6	344.8	PRKCH	218764_at
296	152.2	104.4	135.5	114.4	139.2	133.6	245.7	PRKCH	1559425_at
297	464.4	382.1	336.2	179.8	179.4	285.6	199.6	PRKCI	213518_at
298	443.8	380.3	272.3	161.4	128.7	147.8	107.8	PRKD1	205880_at
299	129.2	148.2	162.6	85.7	98.7	96.6	86.5	PRKD2	241669_x_at
300	304.3	229.9	355.3	270.2	2072.6	856.3	419.5	PRKX	204060_s_at
301	169.9	131.9	249.6	103.3	1035.7	430.5	216.2	PRKX	204061_at
302	114.7	112.6	96.5	37	40.1	40.3	34.2	PRO1853	230379_x_at
303	192.7	162.9	252.3	213.1	309	432.8	306.5	PSEN1	207782_s_at
304	107.6	106.7	87.7	31.2	117.7	34	81.7	PTGS2	204748_at
305	157.6	661.3	173.4	811.7	273.1	223.7	289.7	PTTG	203554_x_at
306	34.3	41.4	33.6	91.9	54.5	64.6	56.2	RAB11B	34478_at
307	343.9	449.3	397.2	810.4	721.2	682.2	633.9	RAB1B	220964_s_at
308	1565	2179.5	3214.8	2512.6	752.7	1807.3	4704.6	RBP1	203423_at
309	875.9	656.3	714.3	314.5	268.4	332.7	376.8	RBPSUH	207785_s_at
310	972.4	1253	944.1	755.3	672	625.3	418.4	RDX	212397_at
311	37.3	46.2	53.1	44.3	212	120.4	92.3	RHOU	223169_s_at
312	370.6	691.9	642.2	438.1	1445.1	1134.4	465	RHOU	223168_at
313	160.8	96.5	101	68.2	70.9	75.8	78.9	RPL19	243005_at
314	298.5	415.5	204.5	247.3	151.4	307.8	195.7	RPS6KA2	212912_at
315	81.3	86.4	85.8	134.2	107.7	151.2	134.4	RPS6KA2	1557970_s_at
316	302.8	242.8	334.7	108.8	100.3	146.4	234.7	RPS6KA3	226335_at

317	104.1	83.9	136.8	82	61	71.5	128.3	RPS6KA3	203843_at
318	410.7	230.2	332.8	201.4	899.7	1376.6	464.7	RPS6KA5	204633_s_at
319	253.3	158.4	208.9	121	430.9	620.8	285	RPS6KA5	204635_at
320	499.2	395.7	431.6	207.9	211.4	240.9	255.7	RPS6KB1	226660_at
321	232.4	195.6	221.5	105.2	105.3	122.4	129.4	RPS6KB1	204171_at
322	87.1	51.2	46.9	62.6	45.7	41.5	132.9	RSPO3	228186_s_at
323	357.8	159.1	270.3	54.1	30.7	40.4	119	RUNX2	232231_at
324	53.8	59.4	63.4	101	95.8	93.9	104.7	SCD	211162_x_at
325	234.2	294.5	311.4	338.6	227.2	237.1	199	SCD	200832_s_at
326	245.3	307.2	323.1	622.6	554.6	566	539.9	SCD	200831_s_at
327	1417.6	1297.1	1174.3	661.1	804.1	524.3	506.4	SDC2	212158_at
328	373.4	333.6	322.1	269.3	236	205.2	193	SDC2	212157_at
329	401	361.1	434.6	481.1	744	406.3	439.4	SDC2	212154_at
330	1220	3009.1	717.5	3095.3	274.4	252.5	111.7	SFRP1	202037_s_at
331	38.6	69.3	33.4	222.5	35.5	32.3	30	SFRP1	202035_s_at
332	284.6	664.2	165.9	2260.2	231.2	196.1	78.4	SFRP1	202036_s_at
333	172.2	250.8	188.3	201.1	144.3	124.7	96.9	SFRP4	240297_at
334	1390.6	7020.5	1409.4	3921.8	286.7	76	58.6	SFRP4	204052_s_at
335	3841.6	12206.9	3922.9	10468	1313.4	453.9	253	SFRP4	204051_s_at
336	158.1	462.7	188.1	435.4	245.1	237.7	189.9	SLC9A3R1	201349_at
337	62.8	132.7	66.7	133.1	91.8	59.4	67.9	SMC2	204240_s_at
338	319.6	200.4	446.3	155.4	608.2	485.3	666.3	SNX9	223027_at
339	340.2	191.3	420.2	211.2	858.8	550.2	743.9	SNX9	223028_s_at
340	45.1	30	46.4	34.1	41.3	48.8	69.1	SNX9	215284_at
341	180.9	208.2	212.2	45.7	37	32.9	32.9	SOSTDC1	213456_at
342	646.5	76.4	214.9	47.9	34.9	62.5	109	SOX9	202935_s_at
343	664.5	102	296.2	74.7	73.1	101.8	125.4	SOX9	202936_s_at
344	2114.2	2849.8	3444.2	1732.3	2144.3	2051.9	1255.2	SPARC	212667_at
345	148.2	201.1	282.6	350.2	185.1	239.7	264.8	STRA6	221701_s_at
346	1790	1507.5	1642.3	690.5	369.6	401.5	1003	TACSTD2	202286_s_at
347	84.4	72.5	108.6	71.1	99.8	159	130.7	TCF2	208135_at
348	395.5	350.4	483.7	447.2	489	1214.9	742.7	TCF2	205313_at
349	56	44.4	57.6	54.2	50.7	145.1	81.7	TCF2	240935_at
350	484.5	702	398.3	491.2	258	219.7	204.6	TCF3	209153_s_at
351	62	100.7	63	104.6	43.8	56.5	58.7	TCF3	209152_s_at
352	210	210.8	152.5	279.4	155.2	137.4	121	TCF3	221016_s_at
353	254.8	204.8	146.9	85.8	71	47.1	89.5	TCF4	215164_at
354	1152.3	880.8	722.1	349.2	166.9	143.9	228.1	TCF4	222146_s_at
355	1449.2	1101.5	931.1	523.1	211	185.4	249.5	TCF4	203753_at
356	64.6	46.3	43	37.2	41.9	35.6	53.4	TCF4	244480_at
357	666.2	422.5	645	194.1	416	363	348.9	TCF7/L2	216035_x_at
358	501	332.8	490.5	155.4	352.9	310.7	284.8	TCF7/L2	216037_x_at
359	444.6	248.5	427.4	144.1	299.7	280	246.7	TCF7/L2	216511_s_at
360	2287.5	1404.5	2002.6	786.6	1664.3	1436.8	1231.3	TCF7/L2	212761_at
361	172.1	106.6	151.5	82.1	121.9	113.3	124.9	TCF7/L2	232522_at
362	105	169.3	130	292.3	116.3	153.6	311.6	TGFB1	203085_s_at
363	212.2	223.8	191	146	121.7	180.1	254.4	TIAM1	213135_at
364	258	260.4	320	149.9	627.5	329.5	157.8	TLE1	228284_at
365	238.4	285.6	262.4	215.7	552.5	388.5	120.2	TLE1	203222_s_at
366	1162.3	1234.8	1190.8	1060.1	2361.7	1582.7	628.5	TLE1	203221_at
367	140.4	133.5	120.2	123.6	308.3	153.5	86.8	TLE1	215802_at
368	86.8	97.5	98	119.9	244.9	177.5	85.1	TLE1	203220_s_at
369	977	880.7	633.5	858.6	467.8	410.5	571.5	TMPO	224944_at
370	217.3	293.1	171.1	238.4	138.4	103.7	141.4	TMPO	203432_at
371	85.6	67.7	74.3	116.3	65.8	57.4	64.3	TMPO	209754_s_at
372	58.7	51.7	57.8	112.7	68.2	66.1	92.4	TMPO	209753_s_at
373	1100.9	517.3	412	146.8	108.7	31.1	71.3	TNFRSF19	227812_at
374	236.6	384.7	351.9	139.4	129.8	182.9	621.9	TWIST1	213943_at
375	132.6	157.4	228	192.8	99.7	136.1	399.6	UPAR	210845_s_at
376	109.3	137.6	185.2	169.6	103.6	118.1	325.5	UPAR	211924_s_at
377	1363.9	1567.6	2176.8	1153.9	1074.6	924.5	860.4	VCL	200931_s_at
378	748.5	1256.6	880.3	616.1	404.4	910.2	988.3	VEGF	210512_s_at
379	76.3	130.1	113.4	157.8	105.3	251	302.7	VEGF	211527_x_at
380	117.1	151.5	148.1	195	138.7	261.2	278	VEGF	210513_s_at
381	210.4	111.2	176	88.8	185.7	146.3	112.7	VEGFC	209946_at
382	872.1	648.1	532	332.1	299.8	499.6	417.1	WEE1	212533_at
383	130.2	86.5	99.2	144.2	107.1	165.4	141.4	WEE1	215711_s_at
384	657.1	258.5	178	42.9	32.7	30	30	WIF1	204712_at
385	75.3	72.2	79.1	137.7	124.4	112.2	139.6	WNT10B	206213_at
386	1102.8	791.9	1266.4	485.2	320.9	327.2	447.3	WNT2	205648_at
387	89.6	234	204.6	246	119.5	212.1	160.1	WNT4	208606_s_at
388	2640.9	2395.7	3830	991.3	502.3	482.1	597.6	WNT5A	213425_at
389	2515.5	2277.5	3874.7	1577.4	759.4	802.6	1161.6	WNT5A	205990_s_at
390	130	118.2	121.2	99.6	58.9	112.2	115	WNT5B	221029_s_at
391	186.7	178.6	228	363.1	282.1	315.6	372	WNT6	71933_at
392	173.8	134	147.8	144	86.2	91.8	88.1	WNT7A	210248_at
393	57	53	58	109.9	144	101.4	96.9	YY1	213494_s_at



Expression of Wnt/β-catenin pathway targets and components during steroid hormone treatment and during the menstrual cycle. Microarray data of a number of relevant Wnt/β-catenin pathway components and targets detected by specific probesets are plotted. For some genes more probesets were available, and therefore more sets of data are plotted in one figure (different colours). On the y-axis the normalized expression levels x 1000 are indicated, on the x-axis the different experimental groups are indicated: 1= non treated, 2= E2 treated, 3= E2 + MPA treated (present data for postmenopausal 21-day steroid hormone treated patients), a= proliferative endometrium, b = early secretory endometrium, c = mid secretory endometrium, d = late secretory endometrium (data from (Talbi, 2006)

4

IDENTIFICATION OF QUIESCENT, STEM-LIKE CELLS IN THE DISTAL FEMALE REPRODUCTIVE TRACT

Wang Y, Sacchetti A, van Dijk MR, van der Zee M, van der Horst PH, Joosten R, Burger CW, Grootegoed JA, Blok LJ, and Fodde R.

ABSTRACT

In fertile women, the endometrium undergoes regular cycles of tissue build-up and regression. It is likely that uterine stem cells are involved in this remarkable turn over. The main goal of our current investigations was to identify (quiescent) endometrial stem cells by means of a pulse-chase approach to selectively earmark, prospectively isolate, and characterize label-retaining cells (LRCs). To this aim, transgenic mice expressing the fusion protein histone 2B - GFP (H2B-GFP) in a Tet-inducible fashion were employed. After 7 days of doxycycline treatment (pulse), the doxycycline water was replaced by normal water (chase). Over time, dividing cells progressively loose the GFP signal after each mitosis, whereas quiescent (infrequently dividing) cells will retain H2B-GFP expression. We evaluated H2B-GFP retaining cells at different chase time points and identified long-term ($\geq$12 weeks) LRCs. These LT-LRCs do not express estrogen receptors and a low level of progesterone receptors (ER^- and PR^-), which is reminiscent of stem cells in other hormone-regulated organs such as the mammary gland. FACSorted LRCs are able to form spheroids capable of self-renewal and differentiation. Under serum stimulation spheroid cells can form glandular structures *in vitro* which express markers of mature Műllerian epithelial cells. In summary, it is shown that quiescent cells located in the distal oviduct have stem-like properties and can differentiate into distinct cell lineages specific of endometrium, proximal and distal oviduct. Future lineage-tracing studies will be needed to elucidate the role played by these cells in homeostasis, tissue injury and cancer of the female reproductive tract in the mouse and eventually in man.

INTRODUCTION

Stem cells are relatively undifferentiated and naive cells endorsed with the ability to self-renew and to give rise to committed progenitors and eventually to differentiated cell lineages. Somatic stem cell niches such as skin[1-4], mammary gland[5], stomach[6, 7], and bone marrow[8, 9] have been shown to encompass both quiescent and cycling populations. Whereas cycling stem cells maintain daily homeostasis, their quiescent equivalents have been postulated to play a rate-limiting role in tissue regeneration upon injury[10, 11]. To date, very little is known about the nature and localization of stem cells in the female reproductive tract and in particular in the uterus[12].

The very first evidence for the existence of a stem cell population in the endometrium came by plating out single endometrial cells *in vitro* to assay their clonogenicity. It was observed that 0.08% of epithelial and 0.02% of stromal cells formed large colonies which could be passaged several times[13]. Furthermore, passaging of epithelial colonies in matrigel resulted in large cytokeratin expressing gland-like structures[14]. Additional studies on the identification of endometrial stem cells indicated that, in bone-marrow transplanted patients, donor-derived bone marrow cells could be found in the endometrial epithelium (0.2 – 48% of cells) and stroma (0.3 – 52% of cells)[15]. This observation was confirmed in lethally irradiated female mice transplanted with bone marrow from male donors: more than 0.5% of the epithelial endometrial cells were shown to be donor-derived[16, 17].

Bromodeoxyuridine (BrdU) label retention also was employed to identify infrequently dividing cells in the endometrium, a feature of stem cells: after 8 weeks of chase approximately 3% of endometrial epithelial cells still retained the BrdU label[18]. After 12 weeks of chase, however, no label-retaining cells (LRCs) were present in the luminal and glandular epithelium, whereas few stromal BrdU-positive cells were observed at the endometrial-myometrial junction in close proximity to the blood vessels. However, DNA-labeling by BrdU is dependent on cell division and in itself represents a genotoxic insult for quiescent stem cells, thus triggering their cell cycle activation[8]. Here, we have employed a non-mutagenic and cell cycle independent approach, namely *in vivo* pulse-chase with the histone 2B - green fluorescent protein (H2B-GFP)[1, 8, 9], towards the identification and prospective isolation of long-term LRCs (LT-LRCs) in the mouse female reproductive tract. We show that *ex-vivo* culture of LT-LRCs sorted from the distal oviduct gives rise to undifferentiated spheroids which display self-renewal capacity and can be induced to differentiate into cells resembling different derivatives of the female embryonal reproductive tract, the Müllerian duct.

MATERIALS AND METHODS

Mice

Transgenic HNRPA2B1/rtTA2S-M2 (rtTA) mice[19] were bred with tetO-HIST1H2BJ/GFP (H2BGFP) mice[1] (kindly provided by Elaine Fuchs, New York). Young adult (8 – 12 weeks of age) compound heterozygous rtTA/H2BGFP transgenic mice and control littermates were administered doxycycline (Sigma, Zwijndrecht, the Netherlands) at 2 mg/ml in 5% sucrose containing drinking water for 7 days (pulse). Doxycycline-containing water was protected from light and refreshed every 2 days. After doxycycline treatment, doxycycline-containing water was replaced by normal water (chase). GFP expression in mice was analyzed at various time points after doxycycline treatment, from 0 till 47 weeks. All experiments conducted with mice were approved by local animal ethics committee (DEC permit numbers EUR1494 and EUR1785) and were in accordance to international guidelines and regulations.

Antibodies and chemicals

Primary antibodies were: biotinylated anti-mouse CD31 (BD, Breda, the Netherlands), biotinylated anti-mouse CD45 (BD), biotinylated anti-mouse TER-119 (BD), anti-CD44 (PE) (BD), rabbit anti-GFP (Invitrogen, Breda, the Netherlands), mouse anti-GFP (Roche, Woerden, the Netherlands), anti-PR (DAKO, Enschede, the Netherlands), anti-β-catenin (Epitomics, Burlingame, USA), anti-ERalpha (Millipore, Amsterdam, the Netherlands), anti-Keratin 8 (Covance, Uden, the Netherlands), anti- Ki67 (Monosan, Uden, the Netherlands), anti-PAEP (Sigma).

Second antibodies were: rabbit/mouse EnVision (DAKO), anti-rabbit immunoglobulins/biotinylated (DAKO), anti-mouse immunoglobulins/biotinylated (DAKO), anti-rat immunoglobulins/biotinylated (DAKO), anti-goat immunoglobulins/biotinylated (DAKO), anti-rat Alexa Fluor 633 (Invitrogen), anti-rat Alexa Fluor Cy3 (Invitrogen), anti-mouse Alexa Fluor 488, 594 (Invitrogen), anti-rabbit Alexa fluor 594 (Invitrogen), fluorescent anti-rabbit (Invitrogen), Strepavidin-PerCP-Cy5.5 (BD), Streptavidin peroxidase (BioGenex, Duiven, the Netherlands). For nuclear staining, the following were employed: Hoechst 33342, 34580 (Invitrogen), DAPI (Invitrogen), DRAQ5 (Biostatus, Leicestershire, UK).

Employed chemicals and biologicals included: PBS (Invitrogen), Matrigel Matrix (BD), DMEM/F12 (Invitrogen), Antibiotic/Antimycotic (Invitrogen), Gentamycin (invitrogen),

Recombinant Mouse EGF (Invitrogen), Recombinant mouse bFGF (Invitrogen), B27 (Invitrogen), DNAse (Sigma), Collagenase (Sigma), TrypLE express (Invitrogen), Triton (Sigma), Formaldehyde (Merck, Schiphol-Rijk, the Netherlands), Select Agar (Invitrogen).

Flow cytometry

The oviducts of mice chased for 12 weeks were mechanically (Stomacher® 80 microBiomaster, Seward Limited, West Sussex, UK) and enzymatically dissociated in medium containing 3 mg/ml Collagenase (Sigma) and 50 µg/ml DNAse (Sigma) at 37°C, in a 5% CO_2 incubator for 30 minutes in order to obtain a single cell suspension. To remove debris, the cell suspension was washed in PBS and passed through a 40 µm cell strainer (BD). To exclude non-epithelial cells from analysis, cells were stained for different lineage markers with primary antibodies directed against CD31, CD45 and TER-119, and Strepavidin-PerCP-Cy5.5 as a secondary antibody. Cells were incubated with 0.2 µg/ml Hoechst-33342 in PBS for 5 minutes to discriminate live from dead cells. Live cells were sorted for high (GFP^+) or very low GFP expression (GFP-negative) by using a FACSAria (BD) flow sorter.

In vitro cell culture and differentiation assay

After sorting, the cells were washed in cold PBS, re-suspended in Matrigel, seeded in 24-well plates (Corning B.V, Amsterdam, the Netherlands), and subsequently covered with culture medium. The culture medium consisted of DMEM/F12 supplemented with 1% v/v B27, 20ng/ml bFGF, 20ng/ml EGF, 100µg/ml Gentamycin in the presence of antibiotics and antimycotics. The medium was refreshed every two days.

For the single cell self-renewal assay, FACSorted GFP^+ and GFP-negative cells were allowed to form spheroids for 20 days. Subsequently, the spheroids were washed in cold culture medium and digested in TrypLE express medium for 5 minutes at 37°C, in a 5% CO2 incubator. The resulting single cell suspension was microscopically evaluated, washed in culture medium, resuspended in Matrigel, seeded in a 24-well plate, covered with culture medium, and allowed to form spheroids again. After 20 days this protocol was repeated to again obtain single cells which formed spheroids in culture.

In order to obtain detailed microscopic images of the spheroids, 1 nm doxycycline was used to induce GFP expression in all cells and facilitate their visualization. Images were captured by confocal microscopy LSM510 (Zeiss, Göttingen, Germany). Three-dimensional reconstruction was performed using the LSM510 software (Zeiss) and Amira (Visage, Berlin, Germany) software packages.

For the differentiation assay, spheroids cultured for five weeks were employed. The spheroids were re-suspended in cold culture medium and plated in tissue culture coated 24-well plates. After 4 hours of culture at 5% CO_2 at 37°C, the medium was carefully removed, and a drop of Matrigel was placed on the top of the spheroids. Subsequently, culture medium supplemented with 10% FCS was added to the Matrigel covering the spheroids to stimulate differentiation. Morphology changes were recorded by confocal microscopy.

Histology and image analysis

Histological analyses were performed on whole mount spheroids and paraffin-embedded sections of spheroids and mouse tissues. For whole mount staining, the spheroids growing in Matrigel were fixed and permeabilized with 4% formaldyhyde and 1% Triton for 1.5 hours at room temperature, and incubated for 30 minutes with blocking solution (3% BSA; 0.1% Triton). Primary antibodies diluted in blocking solution were then incubated o/n, followed by incubation with the appropriate secondary antibody and subsequent substrate incubation.

To embed morphologically intact spheroids in paraffin, they were first fixed in 4% formaldehyde at 37°C for 2 hours before being transferred to fresh 4% formaldehyde at 4°C overnight. Subsequently, fixed spheroids were transferred into 5% Select Agar which was embedded in paraffin to support sectioning.

Immunohistochemical staining for ER, PR, KI67, CD44, GFP, PAEP, CK8 and beta-catenin was performed as described before[20]. For the double fluorescent staining of ER, PR, KI67, CD44 and GFP, the primary antibodies were added simultaneously. The second antibodies were chosen based on the primary antibodies. Nuclei were stained with Hoechst 34580, DAPI or DRAQ5.

The images were acquired by using Axioplan 2 (Zeiss), Olympus BX41 (Olympus, Hamburg, Germany) and Nanozoomer Digital Pathology (NDP) (Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany).

RESULTS

Identification and characterization of LT-LRCs in the distal oviduct

To investigate the origin of stem-like cells in the female reproductive tract, we employed a mouse model that allows the identification and prospective isolation of label-

retaining cells in a non-mutagenic and cell cycle independent fashion, namely with histone 2B - green fluorescent protein (H2B-GFP) as label[1, 8, 9]. To this aim, HNRPA2B1/rtTA2S-M2[19] (rtTA) mice were bred with tetO-HIST1H2BJ/GFP (H2B-GFP) mice[1] and compound heterozygous animals were treated for 7 days by doxycycline administration in drinking water to induce expression of the H2B-GFP label (pulse). H2B-GFP labeling of the vast majority of uterine cells was observed after 7 days of doxycycline pulse both by immunohistochemistry (IHC; Fig. 1) and immunofluorescence (Supplementary Fig. 1A, 0w). Notably, the H2B signal appears much higher in epithelial than in stromal or myometrial cells. In Figure 1, IHC analysis of H2B-GFP after 7 days of doxycycline treatment shows clear and complete epithelial staining in the distal and proximal oviduct, and in the endometrium (Fig. 1B-D; first column). Upon doxycycline withdrawal (chase), dividing cells progressively lose their H2B-GFP signal, while quiescent or infrequently dividing cells will retain the label for longer chase periods (Fig. 1A). In the endometrium, epithelial cells appear to completely loose H2B-GFP expression within 2 to 4 weeks (Supplementary Fig. 1B-C, arrows point to LRCs), whereas stromal LRCs loose H2B-GFP expression between 8 and 12 weeks of chase (Supplementary Fig. 1C, arrows point to LRCs). In the proximal oviduct no label retaining cells are retained after 12 weeks of chase (Fig. 1C). Remarkably however, many LRCs are observed after 12 weeks of chase in the distal oviduct (Fig. 1B, Supplementary Fig 2). Furthermore, after an extensive 47 week chase multiple LRCs are still present in the distal oviduct (Fig. 1B). Here, we will refer to LRCs persisting for at least 12 weeks of chase and onwards, as long-term label-retaining cells (LT-LRCs).

These results were confirmed by FACSorting of GFP^+ cells from whole oviducts before pulse (untreated animals as negative controls) and at chase times 0, 12 and 47 weeks (Fig. 1E). Oviductal cells from untreated mice showed a very low GFP signal (black line). In contrast, after 1 week of dox-pulse (chase time 0 weeks, red line) a large portion of cells displayed a strong GFP signal. At increasing chase times, oviductal cells show substantial loss of their GFP signal (dark-green line; chase time 12 weeks), whereas after 47 weeks of chase only very few GFP^+ cells are present (Fig. 1E, light-green line). Based on the above, all subsequent analyses were performed at 12 weeks of chase.

To characterize the newly identified LT-LRCs in the distal oviduct, IF analysis was performed for expression of estrogen and progesterone receptors, CD44, a stem cell marker in the mammary and other epithelial niches[21, 22], and ki67 as an indicator of proliferative activity. In the mammary gland, epithelial cells are both estrogen and progesterone receptor positive, yet the stem cells do not express either receptors[23-25.]

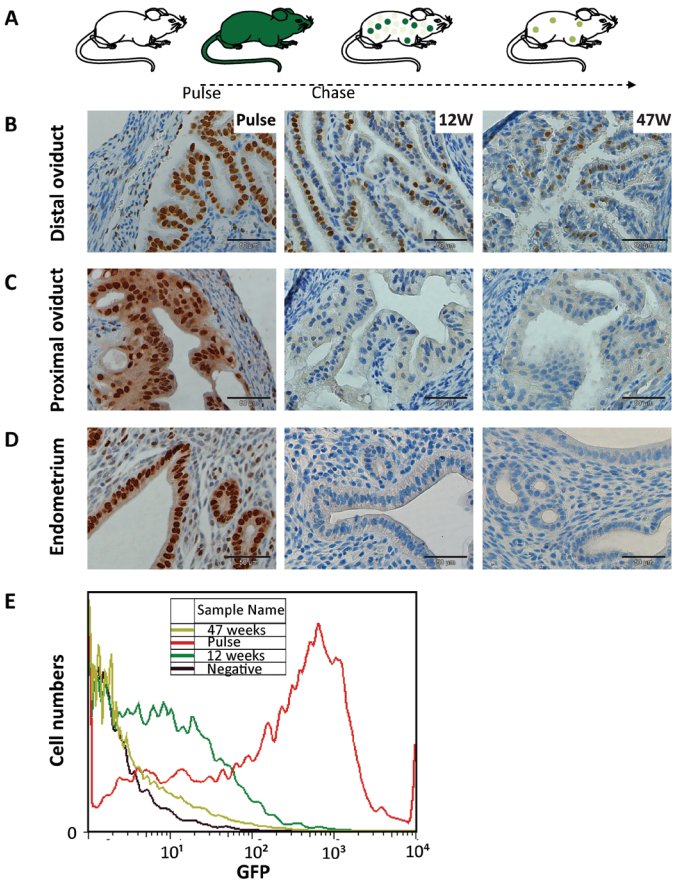


Figure 1. Pulse-chase experiment using the doxycycline-inducible H2B-GFP system. A schematic representation of the experiment is given in A, where treatment with doxycycline results in expression of H2B-GFP throughout the entire body. At different chase points (12 weeks (12W) and 47 weeks (47W)), the GFP signal is progressively lost from dividing cells and retained in quiescent cells in the distal oviduct (B), the proximal oviduct (C) and the endometrium (D). FACSorting was performed on single cell digestions of oviducts from different mice. In the graph, the GFP signal is plotted against the number of cells. Animals used were: untreated mice as negative controls (black line), mice chased for 0 weeks as positive controls (red line), mice chased for 12 weeks (green line) and mice chased for 47 weeks (light-green line).

Furthermore, the vast majority of the epithelial cells from the female reproductive tract are known to response to estrogen and progestagen hormones [26]. Expression of estrogen receptor alpha (ERα) and progesterone receptors A and B (PR) was assessed in LT-LRCs (GFP+) after 12 weeks of chase. As shown in Fig. 2A, LT-LRCs do not express ERα. Furthermore, in contrast to the endometrium, the distal oviduct displayed very low levels of PR expression (Fig. 2B and Supplementary Fig. 3) and, accordingly, LT-LRCs

appear negative for PR expression (Fig. 2B). To validate the quiescent nature of LT-LRCs, expression of the proliferation marker Ki67 was also evaluated. Overall, very few ki67-positive cells were observed in the distal oviduct and, accordingly, double positive cells for GFP and Ki67 were never observed (Fig. 2C and Supplementary Fig. 4). As for CD44, many cells throughout the distal oviduct were positive, though never in GFP+ cells (Fig. 2D). Notably, double staining analysis revealed that most CD44-expressing cells also expressed ERα (Fig. 2E).

Overall, these data indicate that a cluster of epithelial LT-LRCs persist within the distal oviduct for up to 47 weeks, whereas other segments of the female reproductive tract in the mouse seem to be characterized by a considerably higher turnover. Notably, LT-LCRs do not express CD44, estrogen and progesterone receptors, and, in confirmation of their quiescent nature, ki67.

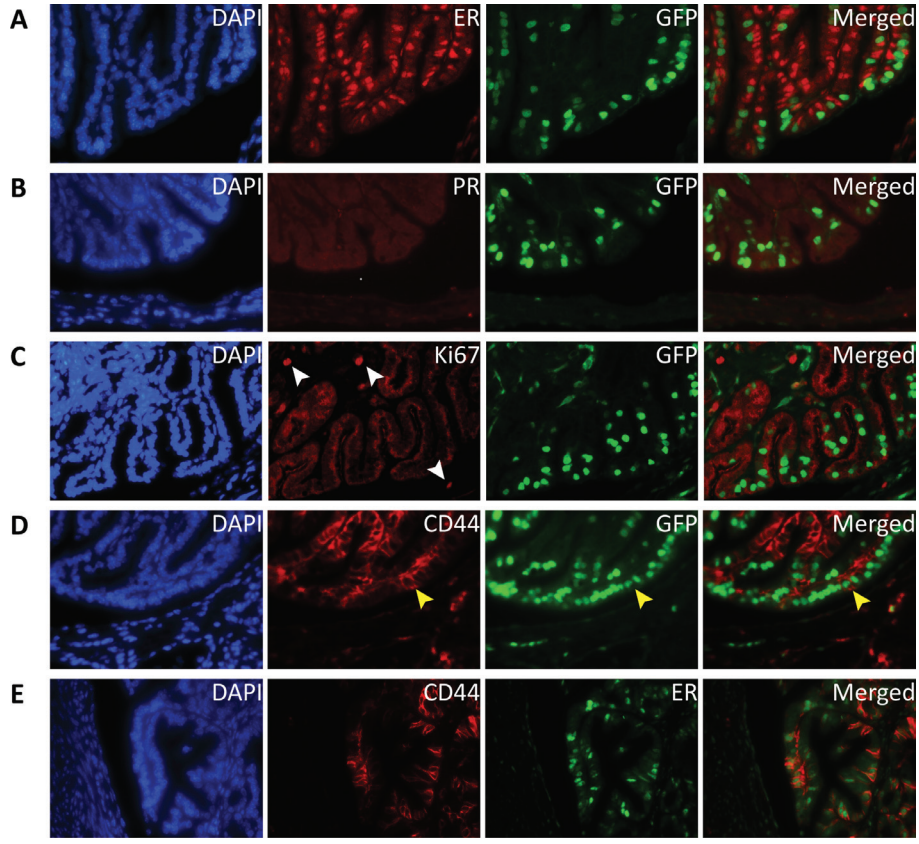
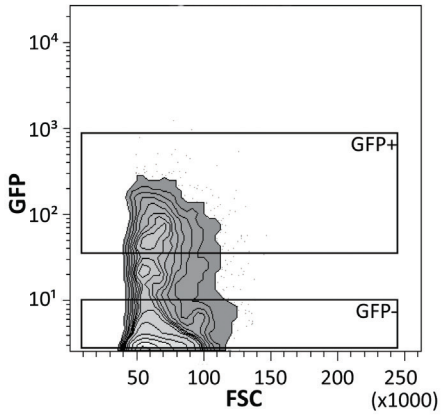


Figure 2. Characterization of identified LT-LRCs (12 week chase) in mouse distal oviduct. Immunofluorescent double-labeling was performed for GFP and ERα (A), GFP and PR (B), GFP and Ki67 (C), GFP and CD44 (D) and CD44 and ERα (E). DAPI staining was performed to display all cell nuclei. White arrowheads indicate Ki67 positive cells, the yellow arrowhead indicates a cell which is positive for CD44 as well as GFP.

A



B

mouse	Spheroid formation per 1000 GFP+ cells	Spheroid formation per 1000 GFP- cells	Secondary spheroids /primary spheroids	Tertiary spheroids /secondary spheroids
#1	2	0.07	32/15	49/19
#2	7.8	0.13	22/4	40/10
#3	5	0	32/11	59/22

C

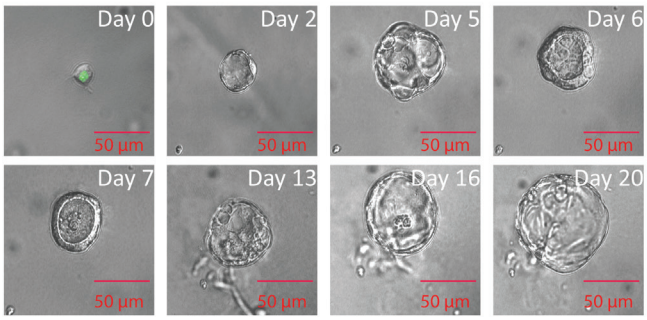


Figure 3. Isolated LT-LRC can form self-renewing spheroids in culture. Oviducts from 12 week chased mice were dissected and a single cell suspension was analyzed by FACS (A). From three different 12 week chased mice, GFP$^+$ and GFP-negative (GFP-) cells were isolated by FACSorting and cultured in Matrigel under serum-free conditions to assay their capacity to form spheroids. Furthermore, when spheroids were formed self-renewal was assayed as indicated in the Materials and Methods section (B). Using limiting dilutions, individual GFP$^+$ cells were cultured for 0 to 20 days in Matrigel under serum-free conditions, thus forming a polarized epithelial organoid (C). The bars in C represent 50 µM.

Ex-vivo isolated long-term LRCs can form epithelial spheroids

When cultured in serum free and in 3D conditions (e.g. matrigel), stem cells are able to form organoid structures often referred to as spheroids. In order to provide indication

of the presence of stem-like cells among the LT-LRCs isolated *ex-vivo* from the distal oviduct, we FACSorted GFP$^+$ and GFP-negative cells from the oviducts of 12-week chased animals (Figure 3A). Sorted single cells were subsequently cultured in Matrigel under serum-free conditions to assay their capacity to form spheroids. As shown in Figure 3B, GFP$^+$ cells form spheroids at a significantly higher rate (1 spheroid/150 cells) than GFP-negative cells (1 spheroid/>7000 cells). Next, enzymatic and mechanic dissociation of the primary organoids was applied in order to obtain a single cell suspension to plate in matrigel and serum-free culture conditions. Secondary spheroids were exclusively obtained from the primary spheroids derived from GFP$^+$ cells though not from those obtained from GFP-negative cells. Likewise, tertiary spheroids were readily obtained from the secondary organoids (Fig. 3B).

Next, we followed single LT-LRCs during the spheroid-formation process. Using limiting dilutions, individual GFP$^+$ cells were first recognized and observed to divide and progressively form an unstructured organoid (between day 2 and 5; Fig. 3C). Over the course of the next few days (day 6-20), early organoids kept growing, simultaneously forming a polarized epithelial-like outer layer of the spheroid (Fig. 3C).

In the female reproductive tract, epithelial cells are, amongst others, characterized by cytoplasmic cytokeratin 8 (CK8) and membrane β-catenin expression. Hence, both the mouse distal oviduct and whole spheroids were analyzed for these markers. As shown in the supplementary Figure 5 the staining pattern in the distal oviduct sections (Supplementary Fig. 5A and 5C) and in the spheroids (Supplementary Fig. 5B and 5D) was similar, confirming the epithelial nature of the LRC-derived organoids (Supplementary Fig. 5).

Under serum-free conditions, spheroids continue to grow slowly but steadily and maintain an undifferentiated appearance for at least 10 weeks. However, upon exposure to serum-supplemented (10% FCS) medium, cell morphology undergoes dramatic modifications (Fig. 4). In order to facilitate the visualization of cell morphology during the differentiation process, spheroids were cultured in the presence of doxycycline and followed by using confocal and phase contrast microscopy and 3D image reconstruction (Fig. 4 and Supplementary Movie 1). Already one day after serum exposure, the formation of multiple cell layers was observed (Fig. 4B and 4C). At day two, monolayers of organoid-derived cells were observed to spread out of spheroids which came in contact with the plastic surface of the culture dish spread (Fig. 4D and 4E). Furthermore, an additional small hollow structure appeared to bud out of the differentiating organoids (Fig. 4D). At day 3, the budded structure appeared as a hollow

tube occasionally flanked by secondary budding structures (Fig. 4F). Subsequently, cells continued to divide, as shown by loss of GFP signaling (after doxycycline withdrawal from the culture medium), with tube elongation proceeding (Fig. 4G). When, in independent experiments, spheroids were allowed to differentiate for 9 (Fig. 4H) and 20 days (Fig. 4I) respectively, formation of more complex structures was observed.

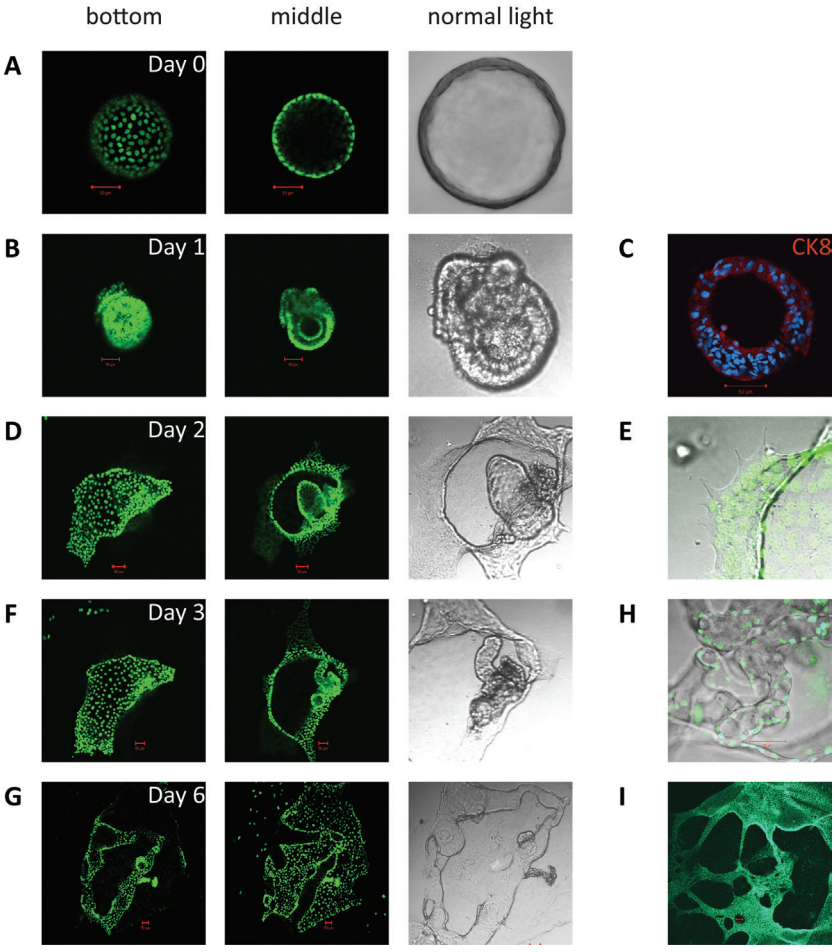


Figure 4. Differentiation of spheroids. 20 day old spheroids derived from oviducts of 12 week chased mice were pulsed for one day with doxycycline and were subsequently allowed to differentiated for 0 (A), 1 (B and C), 2 (D and E), 3 (F) or 6 days (G) in the presence of 10% FCS. The two columns of images on the left hand side of the figure represent confocal images taken at two different planes (bottom and middle). The phase-contrast images in the third colum from the left represents a detail from the confocal image. C represents a spheroid derived from a different animal which was induced to differentiate for 1 day, displaying formation of multiple cell layers as indicated by nuclear DAPI staining and immunofluorescence for CK8. In E attachment of the spheroid to the culture dish is shown. H and I represent spheroids isolated from different animals that were allowed to differentiate for 9 days (H) and for 20 days (I) showing complex structures (nuclei were stained with Hoechst 34580).

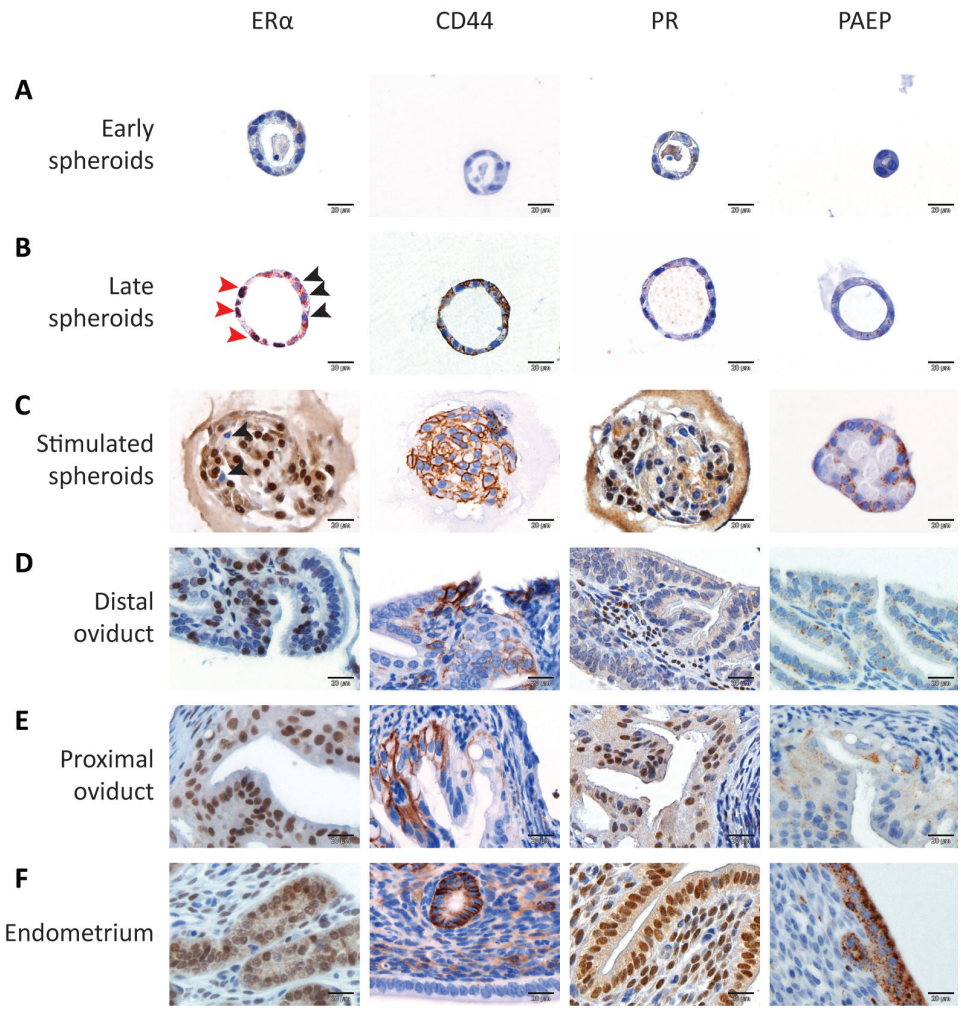


Figure 5. Differentiation of spheroids towards specific cell lineages of the female reproductive tract. Early spheroids (2 days of culture, A), late spheroids (2 weeks of culture, B) and spheroids which were stimulated to differentiated (10% FCS added to the culture medium for 2 days, C) were stained for ERα, CD44, PR and PAEP expression and compared to stained sections from mice distal oviduct (D), proximal oviduct (E) and endometrium (F). The red arrows indicate ERα positive cells, the black arrows indicate ERα negative cells.

In summary, LT-LRCs isolated *ex-vivo* from the distal oviduct can form undifferentiated, self-renewing spheroids which, upon differentiation induction, are able to give rise to complex epithelial structures.

To assess the capacity of the LT-LRC-derived organoids to differentiate towards specific cell lineages of the female reproductive tract, early (1 or 2 days culture), late (2 weeks culture), and more differentiated (see above) spheroids were analyzed by IHC and compared with histological sections from the distal and proximal oviduct, and from

the endometrium for ERα, CD44, PR and PAEP (progestagen-associated endometrial protein, also referred to as glycodelin A) expression (Fig. 5). Whereas in early spheroids all cells were ERα, CD44, PR and PAEP negative (Fig. 5A), late spheroids showed a distinct expression pattern with increased ERα and CD44 expression (though ERα negative cells could still be recognized; Fig. 5B, black arrow). PR and PAEP expression was negative (Fig. 5B). Upon staining of the distal oviduct for the same markers (Fig. 5D) it was observed that the expression pattern of distal oviduct overlapped with that of the late spheroids. Further induction of spheroid differentiation resulted in most cells expressing ERα, PR, CD44, and the uterine marker PAEP (Fig. 5C). Notably, comparison of the expression data between differentiated spheroids and the proximal oviduct and endometrium (Fig. 5E and 5F) revealed marked similarities.

Hence, upon differentiation of spheroids arisen from LT-LRCs, they acquire expression characteristic of cell lineages of the proximal oviduct and endometrium.

DISCUSSION

In this study we employed a H2B-GFP label-retaining assay to identify and isolate quiescent, stem-like cells from the mouse female reproductive tract. Notably, most of the LT-LRCs were observed to cluster within the distal oviduct (including the fimbrial region) where they persist for up to 1 year. In the endometrium, however, only very few and isolated epithelial and stromal LRCs were observed. Previously, by following an analogous approach though based on BrdU labeling, Chan and colleagues also observed a very small population of LRCs in the luminal endometrial epithelium and in the adjacent stroma after an 8-12 weeks chase[18]. As far as the endometrium is concerned, the results presented here are largely in agreement with those by Chan et al. (Supplementary Fig. 1), although in our pulse-chase analysis the glandular epithelium appeared to loose its label at a slower rate than the luminal epithelium, in contrast with the data obtained from BrdU-labeled mice[18] (Supplementary Fig. 1B and 1C). As for the distal oviduct (including the fimbrial region), where a striking number of LRCs was observed to cluster, it was never previously reported as a niche of quiescent cells with stem cell properties. LRCs from this compartment persisted for as long as one year and possibly longer (more extensive chase time-points were not reviewed).

Interestingly, during embryonal development of the Müllerian duct, the anterior region of the coelomic cavity, where *Lim1*$^{+}$ mesoepithelial cells are induced to

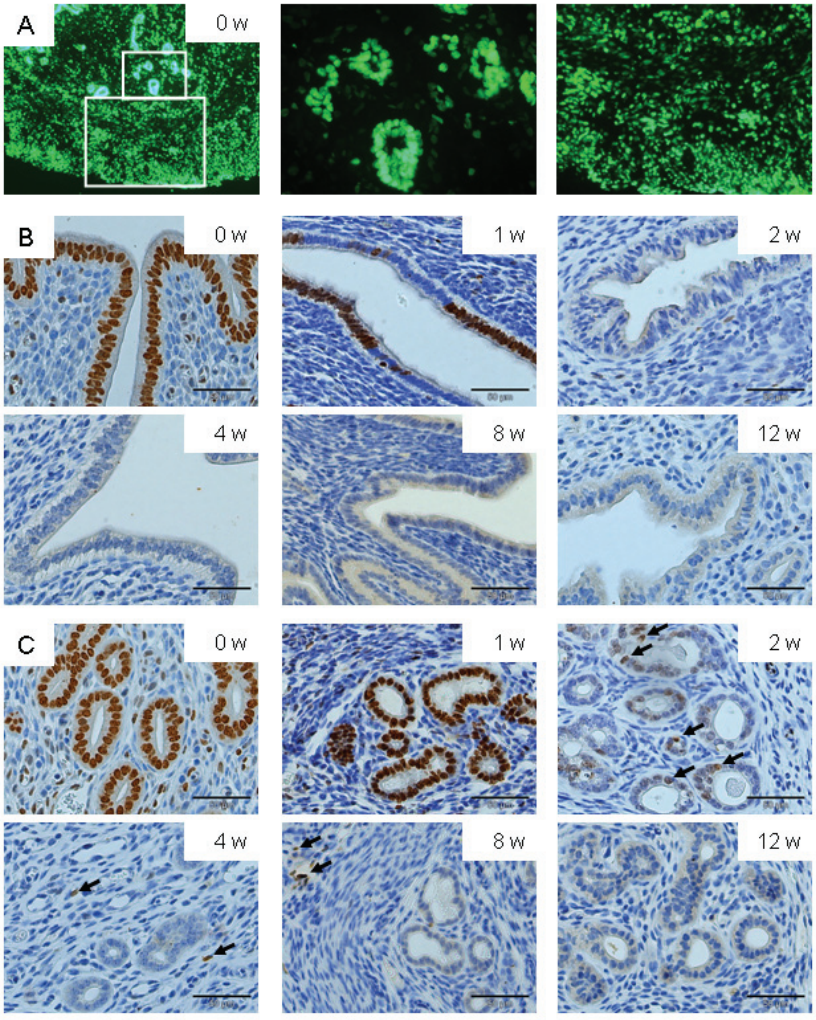
invaginate by Wnt4 expressing cells from the Wolffian duct, gives rise in a later stage to the distal oviduct. This initial invagination elongates alongside the Wolffian duct under the influence of Wnt9b[27, 28] and a population of rapidly proliferating progenitor cells at the tip of the elongation is responsible for the outgrowth which will eventually form the Müllerian ducts[29, 30]. At birth, maturation of the Müllerian duct proceeds with the single layered epithelium differentiating into a stratified squamous epithelium of the anterior vagina and cervix, a simple columnar epithelium of the uterus, and a ciliated or secretory epithelium of the oviducts. Subsequently, mesenchymal cells in close proximity to the epithelial cells differentiate to form stromal cells, while more distant mesenchymal cells differentiate into smooth muscle thus forming the outer muscle layer of the adult Müllerian duct. The last step in female genital tract development is the formation of the glands of the uterus by budding from the luminal epithelium into the uterine stroma followed by extensive branching and coiling.

To characterize the identity of the LT-LRCs in the distal oviduct, FACSorting was employed to prospectively isolate and culture them under conditions which favor outgrowth of self-renewing organoids from clonogenic stem cells. In contrast with H2B-GFP-negative cells, only LRCs were able to generate spheroids in matrigel, serum-free cultures. Moreover, LT-LRC-derived organoids were shown to differentiate in the presence of 10% FCS leading to epithelial cell polarization and the formation of complex, multi-layered structures. IHC analysis of different organoid stadia of the *in vitro* differentiation process revealed that, while early spheroids (2 days of culture) did not express any of the selected endometrium- and oviduct-specific markers in agreement with their stem-like potential (Fig. 5), later stages of spheroid growth (2 weeks in culture) displayed ER and CD44 expression similar to the levels of these distal oviduct markers *in vivo*. Notably, spheroids induced to further differentiate readily displayed expression of all differentiation markers including those specific for the proximal oviduct and endometrium. Hence, our results indicate that the LT-LRCs localized in the distal end of the oviduct are capable of differentiation towards several epithelial lineages of distinct segments of the female reproductive tract in the mouse.

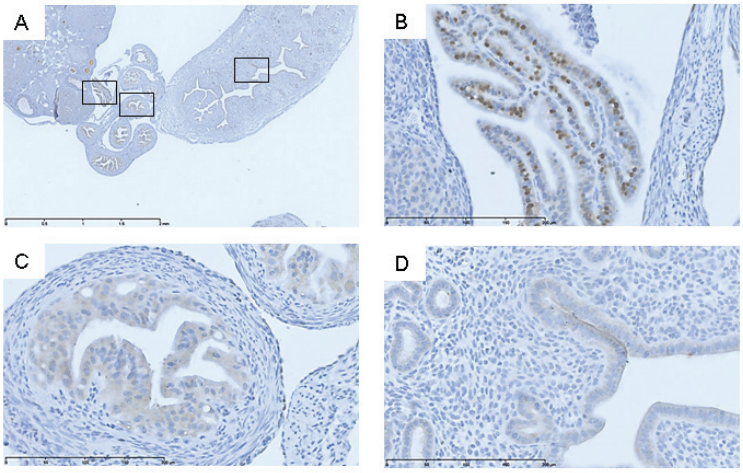
The finding of quiescent, stem-like cells in the distal female reproductive tract (Műllerian duct) could also be relevant for the ongoing discussion on the origin of ovarian cancer [31-33]. In short, there are good indications that some ovarian cancer subtypes do not originate from ovarian surface epithelial cells, but from cells in the distal oviduct. The following observations seem to corroborate this hypothesis: (i) reviewing prophylactically removed adnexa from women with a *BRCA1/2* mutation

showed a high incidence of serous tubal intraepithelial carcinomas in the distal oviduct and fimbriae[34-36]. (ii) The three most prevalent ovarian cancer subtypes are morphologically strongly reminiscent of different Müllerian duct structures (serous ovarian cancer bears likeness to epithelium of the distal oviduct; endometrioid ovarian cancer shows similarity to endometrial glands; and mucinous ovarian cancer resembles the endocervical epithelium[37]). (iii) Well-established ovarian cancer biomarkers such as CA125[38], HE4[39] and PAX2[40], are proteins expressed by endometrial and tubal epithelial cells though not in OSE[38-40]. In view of our findings, an admittedly speculative though stimulating hypothesis can be formulated. Repeated physical and biochemical (inflammatory cytokines and reactive oxygen species present during ovulation) insults may trigger cell cycle entrance and expansion in the otherwise quiescent LT-LRCs in the distal oviduct. Because of the alleged expansion of the LT-LCR compartment upon tissue injury and their differentiation potential, these cells may represent, upon specific (epi)genetic alterations, the cell of origin of distinct ovarian cancer subtypes. Moreover, these cells may also be involved in the development of endometrial benign and malignant aberrations such as endometrial hyperplasia, endometriosis and endometrial cancer.

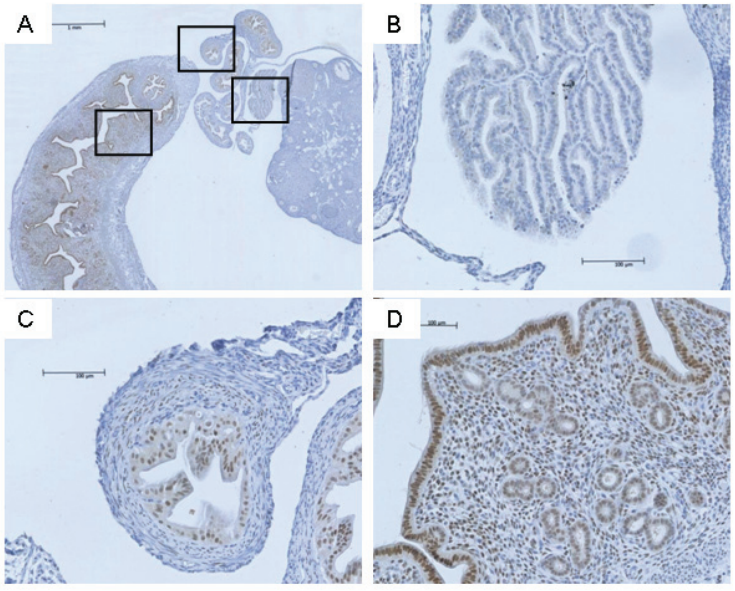
In summary, we show that quiescent cells located in the distal oviduct (including the fimbrial region) have stem-like properties and can differentiate into distinct cell lineages specific of endometrium, proximal and distal oviduct. Future lineage-tracing studies will be needed to elucidate the role played by these cells in homeostasis, tissue injury and cancer of the female reproductive tract in the mouse and eventually in man.



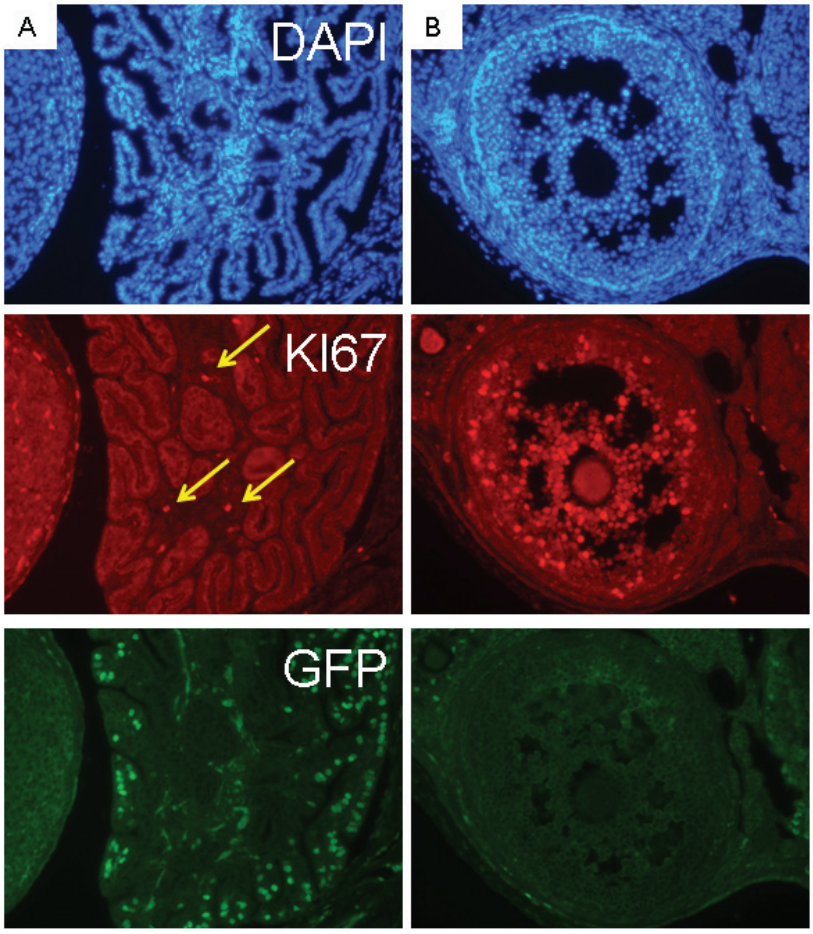
Supplementary Figure 1. Pulse-chase experiment using the doxycycline-inducible H2B-GFP system focusing on the endometrium. Detection of GFP using a fluorescent microscope is shown in A. Details show a strong GFP signal in epithelial cells and a weaker signal in stromal and myometrial cells. Immunohistochemistry for GFP (B+C) was used to detect LRCs in the luminal epithelium (B), or glandular epithelium and stroma (C) in pulsed mice (0 weeks, 0w), and in mice chased for 1 (1w), 2 (2w), 4 (4w), 8 (8w) and 12 weeks. Black arrows point to LRCs in the glandular epithelium at 2 weeks and in the stroma at 4 and 8 weeks.



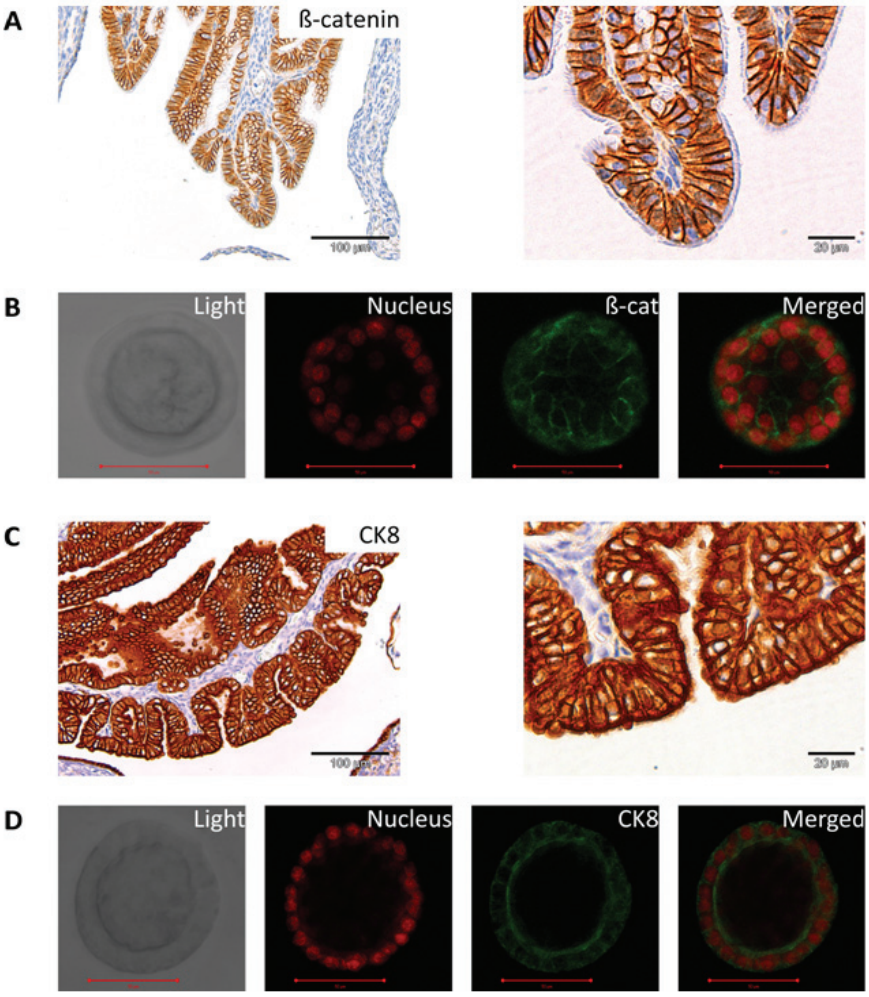
Supplementary Figure 2. Pulse-chase experiment using the doxycycline-inducible H2B-GFP system focusing on GFP signaling in the entire female reproductive tract. After treatment with doxycycline this mouse was chased for 12 weeks before sacrifice. Staining was performed for GFP and in A, a low magnification overview of uterus, oviduct and ovary is shown. Details are shown in B (LT-LRCs in fimbrial region of the distal oviduct), c (proximal oviduct) and D (endometrium).



Supplementary Figure 3. Progesterone receptor expression in the female reproductive tract of a 12 week chased animal. Staining was performed for PR and in A, a low magnification overview of uterus, oviduct and ovary is shown. Details are shown in B (fimbrial region of the distal oviduct), c (proximal oviduct) and D (endometrium).



Supplementary Figure 4. Ki67 staining in the fimbrial region of the distal oviduct (A) and a developing ovarian follicle (B). Yellow arrows indicate rare dividing cells in the fimbrial region of the distal oviduct. Rapidly dividing granulose cells of a developing ovarian follicle are used as a positive control for staining.



Supplementary Figure 5. Expression of epithelial markers β-catenin (A and B) and cytokeratin 8 (CK8, C and D) in the fimbrial region of the distal oviduct (A and C) and in early spheroids (B and D). Oviducts from 12 week chased mice were dissected for immunohistochemistry and for single cell digestion. The single cell digest was FACSorted for GFP^+ cells, which were allowed to form spheroids for 5 days. Two markers, β-catenin (B, green) and CK8 (D, green), were used to stain complete spheroids. The nuclei were contra-stained with DRAQ5 (red). The fluorescent images were captured using confocal microscopy.

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5

GENERAL DISCUSSION

In this thesis, I describe our investigations to assess mechanisms involved in development and homeostasis of female reproductive organs. The research was focussed on molecular and cellular events affecting development and maintenance of Müllerian duct derived organs. At a molecular level, a role for Wnt/β-catenin signaling in maintaining mouse uterine integrity (Chapter 2), and involvement of Wnt/β-catenin signaling in normal and aberrant hormone signaling in the human endometrium (Chapter 3) were demonstrated. At a cellular level, we were able to identify a population of quiescent, stem-like cells located in the mouse distal oviduct (Chapter 4). We anticipate that these new findings, together with other recent observations from us and others, will help to advance basic and clinical research into three important disorders of the female reproductive system: endometrial cancer, endometriosis and ovarian cancer.

Wnt/β-catenin signaling in uterine development

As indicated in the Introduction of this thesis, Wnt/β-catenin signaling affects the uterus both during early embryogenesis and during adult life. For example, Wnt ligands Wnt4 and Wnt9b are important for embryonal outgrowth of the Müllerian ducts[1,2], and Wnt5a and Wnt7a are involved in correct patterning of the Müllerian ducts into upper part of the vagina, the cervix, uterus and oviducts. In the adult, Wnt/β-catenin signaling has a central function in maintenance of a fine balance between proliferation (Wnt/β-catenin signaling has been turned on) and differentiation (Wnt/β-catenin signaling has been turned off).

Constitutive activation of Wnt/β-catenin signaling has been associated with the development of endometrial cancer[3,4]. Over 30% of human endometrial cancers show nuclear β-catenin staining[5,6] which is the hallmark for activation of Wnt/β-catenin signaling. Activation of Wnt/β-catenin signaling can be caused by gene mutations[7] found in components of the degradation complex consisting of APC, AXIN, β-catenin, CK1 and GSK3β. For example, defective APC expression caused by loss of heterozygozity[8], promoter hypermethylation[8], or truncation mutations[9] will prevent formation of the degradation complex which will prohibit degradation of β-catenin, allowing it to travel to the nucleus. Here, nuclear β-catenin will displace the transcription repressor Groucho (TLE), allowing members of the TCF/LEF transcription factor family to modulate the expression of Wnt/β-catenin signaling downstream target genes such as *cyclin D1*, *IGF1* and *CD44*[10,11].

To further clarify the role of constitutive activation of Wnt/β-catenin signaling

during early Müllerian duct development, we generated a novel mouse model in which the Apc gene was conditionaly recombined in mesenchymal cells surrounding the developing Müllerian duct by breeding *Amhr2*$^{Cre/+}$ mice[12] with *Apc*$^{15lox/15lox}$ mice[13]. The rationale behind affecting surrounding mesenchymal cells was that these cells are important in regulating early development of the Müllerian duct through a paracrine mechanism[14].

In order to confirm Wnt/β-catenin signaling activation *in vivo* in our mice, a number of control experiments were conducted. By using an *Amhr2-LacZ* reporter mouse model it was shown that the promoter of *Amhr2* was mainly active in myometrial cells. This finding was further confirmed by breeding *Amhr2Cre* mice with the Rosa26 reporter (R26R)[15] to review Cre expression. In these mice also fairly weak and irregular galactosidase expression was shown in the myometrium. Next, using laser capture microdissection, recombination of the *Apc15lox* allele in *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice was confirmed in the myometrial areas of the uterus. Interestingly, stromal cells, which are thought to arise from the mesenchymal cells surrounding the embryonal Müllerian duct, did not stain for galactosidase activity in the two reporter mice models, nor did these cells display recombination in *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice. It is interesting to know where these adult stromal cells come from, or which cells are the developmental predecessors of adult endometrial stoma cells. Arango et al. (2008) showed in one of their figures that not all cells surrounding Müllerian duct display Amhr2 promoter activity[16]. The stromal cells in adult uterus may originate from these cells. Also, epithelial to mesenchymal transition is a common process during embryogenesis[17], but whether Müllerian duct cells could lose their polarity and be converted into a more mesenchymal phenotype, thus giving rise to uterine stromal cells which do not express Amhr2, remains to be seen.

The most pronounced phenotype for adult *Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ mice is disorganisation or even loss of myometrial muscle fibres, a finding which was confirmed in *Pgr*$^{Cre/+}$;*Apc*$^{15lox/15lox}$ animals. This seems to indicate that Apc knockout does affect development of myometrium. Interestingly, Tanwar et al. (2009) also observed ectopic endometrial tissue within the myometrium of *Amhr2*$^{Cre/+}$;*Ctnnb1*$^{f(Ex3)/+}$ animals[18] and refers to this as resembling a human condition known as adenomyosis (which is characterized by glandular tissue present within the myometrial layer). A question which arises is whether movement of endometrial cells into the myometrium is an active process or passive displacement of tissue to a site where muscular cells have died. According to our results described in Chapter 2, loss of integrity of the myometrium seems to allow for passive movement of endometrial tissue into the muscle layer rather

than that the presence of endometrial cells signifies active invasion.

Recently, Tanwar et al. (2011)[19] published a manuscript dealing with the effects of Wnt/β-catenin signaling in the uterus. Interestingly, the mouse model used by Tanwar et al. (2011) was very comparable to the model used by us[20]. Tanwar et al. (2011) used Amhr2Cre to drive recombination of *Apc* by deletion of exon 14, while in our mice the larger exon 15 of *Apc* was deleted [20]. Similar to our strategy, Tanwar et al. (2011) reviewed activity of Cre by breeding Amhr2Cre mice with LacZ and Yfp reporter mice. Using these reporter mice they could show Amhr2 driven Cre activity in the endometrial stroma; unfortunately they do not mention or show the myometrium. This is in contrast to our observations descripted in Chapter 2 and the observations documented by the original authors of the Amhr2-Cre model[16]. It is possible that the mixed background of the mice which were used by Tanwar et al.[19] lies at the basis of this profound difference (C57BL/6;129/SvEv mixed genetic background). Effects of genetic background on tumorigenesis have been reported in p53-deficient mice[21]. The phenotype of the animals used by Tanwar et al. (2011) partly resembles the phenotype described in our study in Chapter 2 which reported myometrial defects and invasion of endometrial tissue into the myometrium, but Tanwar's (2011) model also shows endometrial cancer, a phenotype which was not observed by us.

Sex hormones and Wnt/β-catenin signaling pathway

The female sex hormones estradiol and progesterone are important in controlling the cyclical renewal of the endometrium. In the first half of a regular menstrual cycle, the proliferative phase, estradiol is required to expand the endometrial layer by inducing cell proliferation, while in the second half of the menstrual cycle, the secretory phase, progesterone levels rise, which antagonizes estradiol's proliferative activity and induces differentiation of the endometrium[22]. Furthermore, increased estrogen signaling and decreased progesterone signaling are both risk factors for endometrial hyperplasia and cancer[23-25]. For this reason, progesterone, i.e. the synthetic medroxyprogesterone acetate (MPA), has been employed in the treatment of advanced and recurrent endometrial cancer (response-rates 15-25%)[26], and in the treatment of well-differentiated endometrial carcinoma in pre-menopausal women that want to preserve fertility (response-rates 60%)[27,28].

Wnt/β-catenin signaling has also been implicated in regulation of the normal menstrual cycle[29,30]. Nuclear β-catenin staining, for example, was observed during the proliferative phase but disappears during the secretory phase of the menstrual cycle[31].

Furthermore, Cloke et al. (2008)[32] showed that knockdown of the progesterone receptor resulted in activation of Wnt/β-catenin signaling in differentiating human endometrial stromal cells. In agreement with this, Satterfield (2008)[33] could show that progestagens, when administered to pregnant sheep, induced a transient decline in Wnt/β-catenin signaling activity. It has also been reported that over 30% of well-differentiated estrogen-associated tumors (Type I endometrioid) show nuclear β-catenin accumulation[5,8]. Moreover, Hou et al. (2004)[34] confirmed that estrogen-induced uterine growth was effectively interfered by the Wnt/β-catenin inhibitor SFRP2 and Jeong et al. (2009)[35] showed that sex hormones could not induce a decidual reaction when β-catenin was dominantly stabilized or conditionally ablated.

Based on these observations, we investigated the hypothesis that progesterone counteracts the proliferative effects of estradiol during the normal menstrual cycle, during hyperplasia and during early endometrial carcinogenesis, by inhibiting Wnt/β-catenin signaling, as described in Chapter 3. According to this hypothesis, sex hormones may modulate Wnt/β-catenin signaling to maintain the balance between proliferation and differentiation.

By comparing gene expression profiles between control, E2 and E2+MPA treated patients[36], a significant number of targets and components of the Wnt/β-catenin signaling pathway were found differentially regulated in the hormone-treated endometrium. Notably, the Wnt/β-catenin signal inhibitor DKK1 and the alleged inhibitor FOXO1 were highly induced by E2 in combination with MPA treatment. This possibly indicates that in the secretory phase of the menstrual cycle, progesterone inhibits Wnt/β-catenin signaling via induction of expression of DKK1 and FOXO1. In literature, it has been reported that DKK1 inhibits Wnt/β-catenin signaling by binding to the Wnt co-receptors LRP5 and LRP6[37], while FOXO1 binds directly to β-catenin[38] thus sequestering the molecule from its normal nuclear activities[39].

By using the well differentiated endometrial cancer cell line Ishikawa, we could show that progesterone up-regulated DKK1 and FOXO1 protein expression, which is in agreement with the gene expression profiles showing that both genes are up-regulated among the E2+MPA treated patients or during the secretory phase of the menstrual cycle and[30]. Furthermore, it was also shown that progesterone effectively inhibited Wnt/β-catenin reporter activity. Moreover, upon DKK1 and FOXO1 knock-down (alone or in combination) by the use of lentiviral sequence-specific shRNAs, inhibition of Wnt/β-catenin signaling by progesterone treatment was partly circumvented.

In summary, we have provided mechanistic evidence of a crosslink between the actions of sex hormones and Wnt/β-catenin signaling in the endometrium. The Wnt/β-catenin signaling inhibitors DKK1 and FOXO1 are two key molecules involved in the process. Our results provide support for the hypothesis that during the proliferative phase of the menstrual cycle increased estradiol levels induce Wnt/β-catenin signaling leading to enhancement of proliferation, while during the secretory phase progesterone levels inhibit Wnt/β-catenin signaling thereby counterbalancing estradiol-induced proliferation and enhancing differentiation.

Stem cells in the female reproductive tract

Stem cells are relatively undifferentiated and naïve cells which have the ability to self-renew and differentiate into progenitor cells of essentially all cell lineages. Adult stem cells for tissues like the skin[40-43], mammary gland[44], stomach[45,46] and the hematopoietic system[47,48] have been identified, but current knowledge on stem cells in the female reproductive tract is limited[49].

A clonogenicity experiment using human endometrial epithelial cells [50] brought the first evidence that endometrial stem cells did exist in the human uterus. In this thesis, we employed a non-mutagenic and cell cycle independent approach, namely in vivo pulse-chase with H2B-GFP, to identify and isolate quiescent, long-term label-retaining stem-like cells (LT-LRCs) from the mouse female reproductive tract. This animal model is an inducible model, in which H2B-GFP is induced when doxycyclin is added to the drinking water. It was observed that LT-LRCs clustered in the distal oviduct. Interestingly, this distal region is also the region where during embryogenesis the initial Müllerian duct anlagen appear, and subsequently, a population of rapidly proliferating progenitor cells is induced to invaginate and the structure elongates alongside the Wolffian duct, eventually to form the Müllerian duct[51,52].

To characterize these newly identified LT-LRCs in the distal oviduct, a double immunofluorescence staining analysis was performed for expression of ERα, PRA/B, CD44 and Ki67. The most significant finding was that in a region which was virtually devoid of PRA/B it was specifically the LT-LRCs which were also negative for ERα. These observations seem to indicate that the LT-LRCs are resistant to sex hormone signaling. For the mammary gland, the presence of ERα and PRA/B negative stem cells has also been reported[53-55]. Notably, although the mammary stem cells were ERα-negative they did respond very well to estrogen signaling. Asselin-Labat et al. (2010)[54] show that ovariectomy reduces the number of mammary stem cells while in mice treated with

oestrogen plus progesterone or in pregnant mice their numbers markedly increase. These authors argue that ERα positive cells in close vicinity of the ERα negative mammary stem cells, upon hormonal stimulation, produce a paracrine factor called RANKL which can bind to its receptor RANK at the surface of ERα negative mammary stem cells. Treatment of mice with anti RANKL monoclonal antibody indeed impaired clonogenicity of the mammary stem cells. In our study, we did observe that ERα positive cells were situated next to the ERα negative LT-LRCs. It is, however, not clear whether the above-mentioned paracrine mechanism is involved in the interaction between the LT-LRCs in the distal oviduct and their ERα positive neighbours.

In order to investigate putative stemness of our LT-LRCs, these cells were isolated by flow cytometry on basis of high retention of H2B-GFP in the nucleus, and cultured under serum-free conditions in matrigel. In contrast to isolated cells which did not express GFP, the LT-LRCs formed undifferentiated structures which are called spheroids. The epithelial origin of spheroids was confirmed by the expression of markers such as CK8 and membranous β-catenin[56-59]. Furthermore, upon digestion of spheroids, individual cells were able to form new spheroids, which, upon repeated digestion again formed new spheroids. This experiment indicates that LT-LRCs have the capacity to self-renew. The most interesting experiment, however, was when spheroids were kept in culture for a longer period of time and were then allowed to differentiate. During the initial weeks of culture it was noted that next to ERα negative cells, ERα-positive cells appeared, and CD44 expression increased over time. This condition seems to resemble the situation in the distal oviduct where ERα/CD44 negative cells (LT-LRCs) reside next to ERα/CD44 positive cells. As depicted in Figure 1, it is hypothesized that these newly produced ERα/CD44-positive cells could function as progenitor cells for different structures of the female reproductive tract. What argues in favour of this hypothesis is that oviduct, uterus, cervix and part of the vagina all develop from the same embryonal structure: the Müllerian duct. Furthermore, as already indicated, embryonal development starts at in the region that later forms the distal oviduct. What argues against this hypothesis is maybe the distance which progenitor cells need to travel before being able to populate the proximal oviduct and endometrium, although one could also argue that if the fertilized oocyte can travel from distal oviduct to the uterus, this might also be possible for an epithelial progenitor cell which is shedded from the distal oviduct.

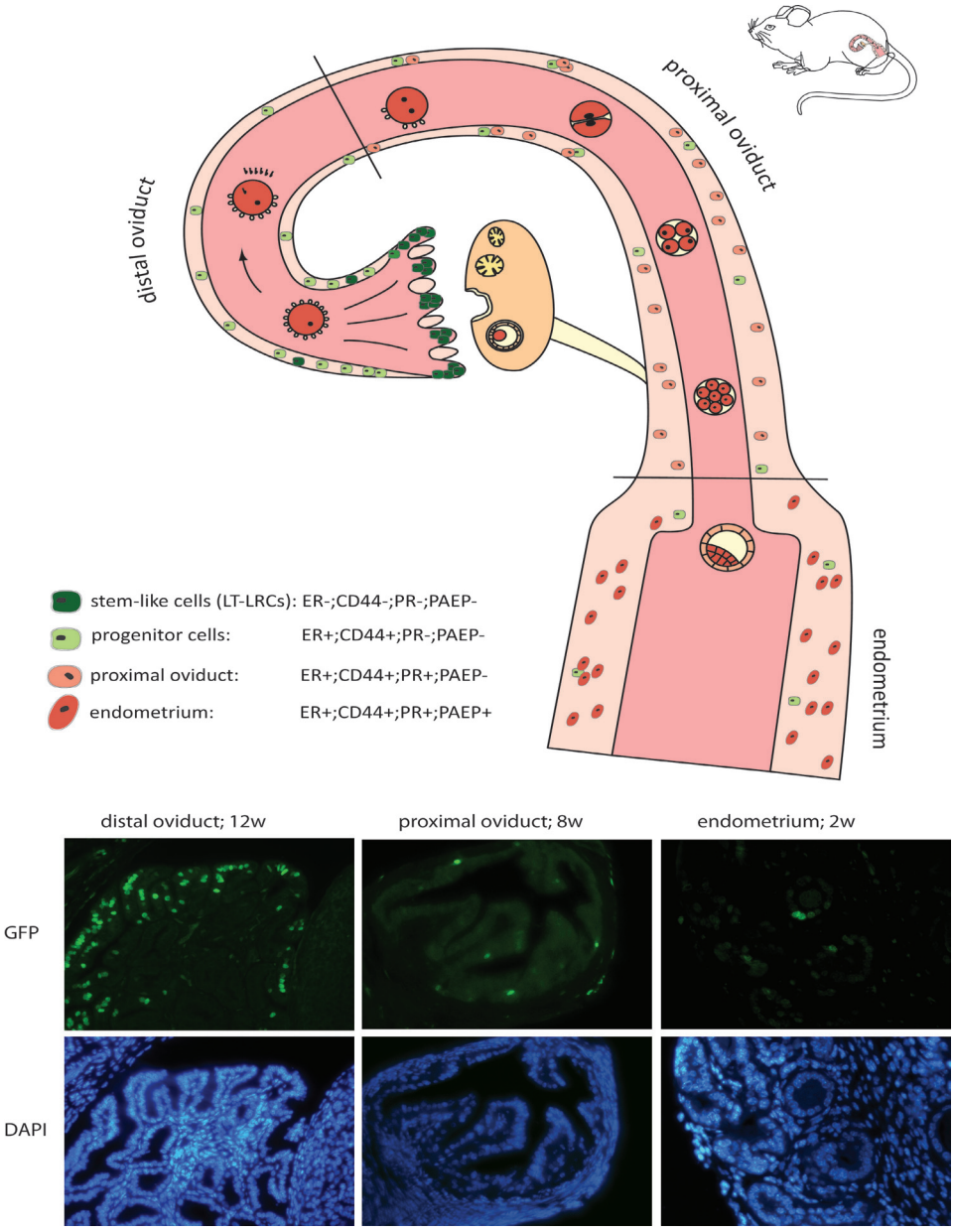

Figure 1. Schematic representation of a hypothesis for epithelial cell lineages formed from LT-LRCs in the distal oviduct. The different cell types are represented in different colors and their specific marker composition is indicated. The LRCs at distal oviduct, proximal oviduct and uterus are shown.

After serum-stimulation, the structure of the spheroids changed dramatically and many cells became positive for ERα, PRA/B and CD44. Furthermore, when we stained for the endometrial differentiation marker PAEP, expression of this marker was also markedly enhanced, indicating similarity between differentiated spheroids and proximal oviduct and endometrium.

On the basis of data obtained so far it is hypothesized that LT-LRCs located in the distal oviduct are stem cells for the epithelial lining of the proximal oviduct and endometrium.

Based on the remarkable location of these cells, the present observations are possibly also of relevance for cancer and in particular ovarian cancer. A prevailing hypothesis on the origin of ovarian cancer argues that ovarian surface epithelial cells (OSEs) become damaged by physical (repeated ovulation) and biochemical insults (inflammatory cytokines and reactive oxygen species present during ovulation). The damaged OSEs may over time become trapped into the ovarian cortical stroma thus forming cortical inclusion cysts. Due to exposure to a new hormone rich environment, epithelial cells lining these cysts undergo metaplasia and eventually progress into dysplasia and ovarian carcinogenesis[60]. Interestingly, endometriosis is also often recognized as a condition predisposing to the formation of ovarian cysts, indicating that Müllerian duct derivatives can become trapped in the ovarian cortical stroma[61-63] and give rise to intra-ovarian endometriosis. However, as far as ovarian cancer is concerned, different observations indicate that, apart from the OSEs, cells originating from the Müllerian duct may be involved[64-66]. First, reviewing prophylactically removed adnexa from women with a BRCA1/2 mutation, several authors showed a high incidence of serous tubal intraepithelial carcinomas in the distal oviduct[67-69]. In sporadic serous ovarian cancer, tubal intraepithelial carcinoma of the distal oviduct and fimbriae was observed in 50% of the cases[70], while in extraovarian peritoneal carcinoma (otherwise indistinguishable from serous ovarian cancer), no involvement of OSEs could be demonstrated. Second, the three most prevalent ovarian cancer subtypes are morphologically strongly reminiscent of different Müllerian duct structures: serous ovarian cancer bears likeness to epithelium of the distal oviduct (the fimbriae); endometrioid ovarian cancer shows similarity to endometrial glands; and mucinous ovarian cancer resembles the endocervical epithelium[65]. Third, well-established ovarian cancer biomarkers such as CA125[71], HE4[72] and PAX2[73], are proteins expressed by both endometrial and tubal epithelial cells but not in OSEs[71-73]. Fourth, in patients with a p53 mutation, the distal oviduct is found as a common site of origin for early serous carcinogenesis[74-76]. p53 is

known to play an important role in maintaining quiescence in hematopoietic stem cells[77] and to control symmetric vs. asymmetric cell division in mammary stem cells[78,79].

In view of our present findings, we would like to propose a new hypothesis on the origin of ovarian cancer. The above-mentioned repeated physical (ovulation) and biochemical (inflammatory cytokines and reactive oxygen species present during ovulation) insults may trigger cell cycle entrance and expansion in the otherwise quiescent LT-LRCs in the distal oviduct. Because of the alleged expansion of the LT-LCRs compartment upon tissue injury and their differentiation potential, these cells may represent the cells of origin of distinct aberrations. Before or after undergoing subsequent genetic or epigenetic alterations, the aberrations could affect different derivatives of the reproductive tract, such as giving rise to endometriosis, early malignant precursors of serous ovarian cancer and possibly other ovarian cancer subtypes.

In conclusion, the quiescent cells identified in the distal oviduct are likely to represent bona fide stem cells. Future lineage-tracing experiments should be addressed to further reveal the role of the LT-LRCs.

Concluding remarks and future perspectives

The overall theme of the present thesis has been to investigate and identify signaling pathways and stem cells in the mammalian female reproductive system. With the observation that activation of Wnt/β-catenin signaling in mesenchymal cells surrounding the Müllerian ducts induced profound myometrial defects, and with the identification of DKK1 and FOXO1 as intermediates of progesterone induced inhibition of Wnt/β-catenin signaling, a central role for Wnt/β-catenin signaling in regulation of uterine homeostasis was established. This work is currently being continued by studying cross-talk between the actions of sex hormones and Wnt/β-catenin signaling in endometrial carcinogenesis.

The study into the identification of quiescent, stem-like cells in the mouse distal female reproductive tract should be considered as a starting point. Much more work is needed before we can be certain that these stem-like cells in the distal oviduct are stem cells of most epithelial derivatives of the Müllerian ducts. Lineage tracing experiments in which we can visualize that cells originating from the distal oviduct can populate the proximal oviduct and endometrium are vital to proof this point. Furthermore, more clinical studies in which the link between aberrations in the distal oviduct and endometriosis and ovarian cancer is made are currently being conducted.

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6

SUMMARY

SAMENVATTING

总结

SUMMARY

During her fertile years, the endometrium of fertile women undergoes regular cycles of regeneration, differentiation and shedding, driven by changing concentrations of the steroid hormones estradiol and progesterone. In the present study, the role of Wnt/β-catenin signaling in relation to steroid hormone signaling and the balance between proliferation and differentiation was investigated. Furthermore, since the consequence of hormone signaling in the endometrium is tissue degradation followed by regeneration, the role of stem cells was also investigated.

Chapter 1, the introductory chapter, reviews what is known about a functional link between steroid hormone signaling, Wnt/β-catenin signaling, and endometrial physiology. In the normal human menstrual cycle, estradiol induces proliferation during the first two weeks, whereas during the third and fourth week of the cycle progesterone inhibits this estradiol-induced proliferation thus stimulating cellular differentiation. The two steroid hormones also play a role in oncogenesis of the endometrium: unbalanced hormone signaling can induce endometrial hyperplasia which may continue to develop into endometrial cancer. Elevated estrogen receptor signaling, caused for example by overweight or use of the synthetic analog tamoxifen, significantly increases the risk of endometrial carcinogenesis.

The role of Wnt signaling in initiation, development and function of the female reproductive tract, investigated, in particular in the mouse, is significant. There are good indications that hormonal regulation of the human menstrual cycle during reproductive life is also mediated by estrogen-induced activation and progesterone-induced inhibition of Wnt/β-catenin signaling. Similar to elevated estradiol signaling, constitutive activation of canonical Wnt signaling has been associated with the development of human endometrial cancer. Activation of Wnt/β-catenin signaling can be caused by mutations that affect β-catenin degradation, in women and in mouse models. These mutations result in β-catenin protein stabilization which, upon translocation to the nucleus, can activate transcription of Wnt/β-catenin target genes.

Chapter 2 describes the role of constitutive activation of Wnt/β-catenin signaling during uterine development using a novel mouse model (*Amhr2*$^{Cre/+}$;*Apc*$^{15lox/15lox}$). It was observed that the promoter of *Amhr2* was mainly active in myometrial cells. Furthermore, PCR results confirmed that recombination of the *Apc*15lox allele occurred exclusively in the myometrial region of the uterus. Consequently, the main phenotype in these *Apc* conditional knock-down mice was loss and aberrant organization of the

myometrial muscle fibers. As a result, endometrial tissue was sometimes observed in the myometrial layer, a phenomenon which in our opinion is a passive rather than an active invasion process. A mouse model to inactivate Wnt/β-catenin signaling specifically in the epithelial cells of the developing Müllerian ducts, unfortunately is not available. Hence, we cannot exclude that there might be an additional role for this signaling pathway, directly targeting the epithelial cells and their derivatives.

As described in **Chapter 3**, it was investigated how progesterone regulates Wnt/β-catenin signaling. Among the genes regulated by progesterone in patients, *DKK1* and *FOXO1* as well as genes encoding other known inhibitors of Wnt/β-catenin signaling, were identified and selected for further investigations. First, it was established that during progesterone-driven inhibition of Wnt/β-catenin signaling, both *DKK1* and *FOXO1* gene expression were induced. Secondly, it was observed that progesterone could not inhibit Wnt/β-catenin signaling in the endometrial cancer cell lines in the absence of DKK1 and FOXO1 (by the use of lentiviral sequence-specific shRNAs). To put the observed results in a clinical perspective, patient samples (hormone treated endometria, hyperplasia and endometrial cancers) were evaluated for Wnt signaling activation using a histochemical approach. On the basis of these results it was concluded that the Wnt inhibitory effect of progesterone action is likely to play a rate-limiting role in the maintenance of endometrial homeostasis and, upon loss of progesterone signaling, in tumor onset and progression towards malignancy.

In **Chapter 4**, the research focused on the identification, characterization and isolation of stem cells of the female reproductive tract is presented. The initial observations were well in line with those published in several papers by the group of Gargett et al. (2004, 2006, 2009), where they reported short-term label-retaining cells in the uterus. In subsequent experiments, we found a new population of long-term label-retaining cells at the distal oviduct. These cells were isolated and were shown to be able to form undifferentiated spheroids in stem cell medium, to self-renew upon re-culture of spheroid cells, and to differentiate into glandular structures which expressed markers of mature Műllerian epithelial cells. Based on these findings, it is suggested that these quiescent, stem-like cells located within the distal oviduct might contribute to the homeostasis of at least part of the female genital tract, in particular concerning the cyclic regeneration of the epithelial lining of the endometrium and the maintenance of the oviduct.

Chapter 5 provides a general discussion, where the significance of the current findings is put into a broader perspective.

SAMENVATTING

Tijdens de vruchtbare jaren van een vrouw ondergaat haar endometrium, onder invloed van wisselende concentraties van de steroïdhormonen oestradiol en progesteron, regelmatige cycli van opbouw, differentiatie en afbraak. In het onderzoek beschreven in dit proefschrift werd de rol van Wnt/β-catenine signalering in relatie tot hormonale regulatie van de balans tussen opbouw (proliferatie van cellen) en differentiatie van het endometrium-weefsel onderzocht. Daarnaast werd, omdat hormoonwerking in het endometrium leidt tot een cyclus van weefsel-afbraak gevolgd door opbouw, de rol van stamcellen bestudeerd.

Hoofdstuk 1, de algemene introductie, beschrijft de functionele relatie tussen steroïdhormoon-signalering, Wnt/β-catenine-signalering en endometrium fysiologie. In de eerste twee weken van een normale menstruele cyclus zorgt oestradiol voor proliferatie van het endometrium. In de derde en vierde week wordt deze oestradiol-geïnduceerde proliferatie vervolgens geremd door progesteron, wat leidt tot stimulatie van cellulaire differentiatie. Daarnaast spelen zowel oestradiol als progesteron een rol in de oncogenese van het endometrium waarbij afwijkende hormoonsignalering kan leiden tot endometriumhyperplasie, welke zich soms kan ontwikkelen tot endometriumkanker. Verder leidt een verhoogde oestradiol receptor-signalering, bijvoorbeeld veroorzaakt door overgewicht of het gebruik van Tamoxifen, tot een significant verhoogd risico op endometriumkanker.
De rol van Wnt/β-catenine signalering tijdens de ontwikkeling en functie van het vrouwelijk genitaal (voornamelijk onderzocht in muizen) is zeer groot. In muizen, alsmede in de humane situatie, zijn er goede aanwijzingen dat de cyclus tijdens de vruchtbare periode gereguleerd wordt door oestradiol-geïnduceerde activering en progesteron-geïnduceerde remming van Wnt/β-catenine signalering. Continue Wnt/β-catenine-signalering is, evenals verhoogde oestradiol-signalering, geassocieerd met het ontstaan van humaan endometrium-carcinoom. In muismodellen en ook in mensen, kan activering van Wnt/β-catenine signalering worden veroorzaakt door mutaties in β-catenine. Deze mutaties leiden tot stabilisatie van het β-catenine eiwit, dat, na translocatie naar de celkern, zorgt voor transcriptie van Wnt/β-catenine gereguleerde genen.

Hoofdstuk 2 beschrijft de rol van continue Wnt/β-catenine signalering tijdens de ontwikkeling van de uterus in een nieuw muismodel (*Amhr2*$^{Cre/+}$*; Apc*$^{15lox/15lox}$), waarmee het mogelijk is om vanaf embryonale dag 12.5 het *Apc* gen (adenomatosis polyposis coli) uit te schakelen, en daarmee Wnt/β-catenine signalering aan te zetten. In dit model werd gevonden dat de promotor van het *Amhr2* gen voornamelijk actief was in de cellen van het myometrium. Verder bevestigde analyse van de hoeveelheid aanwezig mRNA (met de PCR methode) dat recombinatie van het *Apc*15lox allel exclusief plaatsvond in het

myometrium van de uterus. Als gevolg hiervan ontwikkelden deze *Apc* mutant muizen een fenotype gekenmerkt door verlies van myometriumweefsel en een afwijkende structuur van het myometrium. Hierdoor werd soms endometriumweefsel in het myometrium waargenomen, waarschijnlijk ten gevolge van passieve invasie. Echter, omdat een inactiverend Wnt/β-catenine muismodel specifiek voor epitheelcellen van de ontwikkelende Müllerse gang niet beschikbaar is, kunnen we een rol voor Wnt/β-catenine signalering in deze cellen niet uitsluiten.

In **Hoofdstuk 3** werd onderzocht hoe progesteron Wnt/β-catenine-signalering reguleert. Tussen een groot aantal progesteron-gereguleerde genen in het humane endometrium werden de Wnt/β-catenine-signalering-remmende genen *DKK1* en *FOXO1* geïdentificeerd en vervolgens gebruikt in verder onderzoek. Ten eerste werd bewezen dat tijdens progesteron-geinduceerde remming van Wnt/β-catenine-signalering *DKK1* en *FOXO1* tot expressie werden gebracht. Daarnaast werd gevonden dat remming van Wnt/β-catenine-signalering door progesteron niet mogelijk was in endometriumkanker-cellijnen waarin *DKK1* en *FOXO1* afwezig waren (met behulp van sequentie-specifieke lentivirale shRNAs). Om deze gevonden resultaten klinisch te bevestigen werden vervolgens weefsels van patiënten (met hormoon behandeld endometrium, met endometrium hyperplasie of met endometriumkanker) gebruikt voor immunohistochemische analyse van de activiteit van Wnt/β-catenine-signalering. Naar aanleiding van deze resultaten werd geconcludeerd dat remming van Wnt/β-catenine-signalering door progesteron een remmende rol speelt in de endometrium-homeostase en dat verlies van progesteron-signalering kan leiden tot maligne ontaarding van het endometrium.

Hoofdstuk 4 beschrijft de identificatie, karakterisering en isolatie van stamcel-achtige cellen van de vrouwelijke geslachtsgangen. De eerste bevindingen bevestigden eerder werk van Gargett et al. (2004, 2006 en 2009) waarin "label-retaining" cellen van de uterus werden gevonden. Daarnaast werd een nieuwe populatie van zeer lang aanwezig blijvende "label-retaining" cellen geïdentificeerd in de distale oviduct. Deze cellen werden geïsoleerd en bleken in staat om, in speciaal stamcel-medium, ongedifferentieerde spheroïden te vormen, te regenereren en te differentiëren in epitheliale klierachtige structuren met morfologische kenmerken van de Müllerse gangen, waaruit uterus en oviduct tijdens de embryonale ontwikkeling ontstaan. Op basis van deze bevindingen werd gehypothetiseerd dat deze, in de distale oviduct gelokaliseerde, stamcel-achtige cellen bijdragen aan de homeostase van tenminste een deel van de vrouwelijke geslachtsgangen.

Hoofdstuk 5 is een algemene beschouwing waarin de gevonden resultaten en de relevantie hiervan worden bediscussieerd.

总结 (Summary in Chinese)

在正常情况下，生育期女性的子宫内膜有一个周期性的增生、分泌和脱落的变化，这个变化是由于雌激素和孕激素的水平变化引起的。在目前的博士课题中，我着重研究了Wnt/β-catenin信号传导以及它与性激素在月经周期中对子宫的增生和分化起的重要作用。与此同时，本课题还研究了干细胞在子宫再生过程中的作用。

在第一章节中，着重介绍了性激素，Wnt/β-catenin信号传导以及子宫生理学。在月经周期(menstrual cycle)的前两个星期，卵巢分泌的大量的雌激素促进内皮层的增生(proliferation)。在月经周期的第三和第四个星期，黄体(corpus luteum)分泌的孕激素可以抑制雌激素的增生作用并促进子宫内皮的分化(differentiation)。性激素除了可以调节正常的月经循环，在子宫的疾病发展过期中也起到了很重要的作用，性激素平衡的破坏可以促进子宫内皮的不正常增生，甚至可以导致子宫癌。比如，服用他莫昔芬（tamoxifen）以及过于肥胖都可以提高身体的雌激素水平，从而促进子宫内膜癌的发生几率。Wnt信号传导已经被证明在雌性生物中特别是老鼠的性生殖管的生成，发育以及它的正常功能中起着重要的作用。有证据表明，雌激素促进的子宫内膜的增生以及孕激素促进的分化都可以通过Wnt/β-catenin信号传导来介导。与雌激素的功能类似，持续激活Wnt/β-catenin信号也与子宫内膜癌相关。在老鼠以及人身上，已经证明β-catenin的突变可以激活Wnt/β-catenin信号。这些突变可以导致β-catenin更稳定，而不容易被降解，由此β-catenin有机会在核中表达，从而激活Wnt/β-catenin信号传导。

第二章节里，我们用了一个动物模型(Amhr2Cre/+;Apc15lox/15lox)论证了Wnt/β-catenin信号传导的激活在子宫发育中的重要性。我们剔除了这个信号传导上游的一个分子——APC，从而让Wnt信号传导通路始终处于激活状态。我们发现Amhr2的启动子活性主要集中在子宫的肌层中。而且PCR的结果也证明Apc基因的敲除也在肌层中。最后我们发现Apc基因敲除会最终导致肌纤维不正常分布或缺失。作为一个肌层缺失的被动结果，内皮组织有时候会进到肌层中。可惜的是，目前还没有只在子宫内皮激活Wnt/β-catenin信号传导的动物模型。所以我们不能排除Wnt/β-catenin信号传导在内皮中有更多的作用。

在第三章中，证明了孕激素调节Wnt/β-catenin信号传导分子机制。在患者样本中，孕激素调节大量的基因，其中包括DKK1 and FOXO1。有意思的是，这两个分子也是Wnt/β-catenin的抑制因子。首先，在孕激素抑制Wnt/β-catenin信号传导的过程中，DKK1 and FOXO1的表达都有明显的升高。其次，我们证明了，如

果我们通过腺病毒介导的shRNA降低DKK1 and FOXO1的表达，孕激素不能有效的抑制Wnt/β-catenin信号传导。我们进一步在病人样本中也观察到了这个机制。孕激素可以通过抑制Wnt/β-catenin信号传导来维持子宫内皮的稳态。一旦失去孕激素的控制，肿瘤就会慢慢生成。

第四章的研究是关于子宫内皮干细胞发现和分离。为了保护自己，干细胞通常分裂比较慢。我们设计了一个动物模型，通过GFP来标记组蛋白(histone2B-green fluorescent protein, H2B-GFP).这个方法不会对细胞产生伤害，而且可以用强力霉素（doxycycline）来诱导GFP的表达。在这个实验中，我们给老鼠喝带有强力霉素的水，这样GFP就会表达。一个星期后把水换成正常的水。这样，随着细胞的分裂，GFP的信号会越来越弱。因为干细胞分裂很慢，通过这个方法我们可以发现干细胞。我发现在输卵管末端存在这样长期保留GFP的分裂缓慢的细胞（LRC）。我们的这个发现和早期Gargett等的报告相吻合。接下来，我们把细胞分离出来，进行体外干细胞培养，我发现这些细胞具有干细胞的两个特性：自我复制和分化。单个LRC细胞就能形成球状结构（spheroid）,如果用血清来刺激球状结构，它会形成子宫腺形结构，并且开始表达一些只有成熟细胞才具有的分化标志。所有的证据表明，这些在输卵管末端的分裂缓慢的干细胞有可能具有帮助生殖管上皮再生的功能。

第五章，总结了本论文的发现并展望了这些发现的意义。

PHD PROTFOLIO SUMMARY

SUMMARY OF PHD TRAINING AND TEACHING ACTIVITIES

Name PhD student: Yongyi Wang
Erasmus MC Department: Reproduction and Development
Research School: Molecular Medicine

PhD period: 11/01/2007~11/07/2011
Promotor(s): J.A. Grootegoed & R. Fodde
Supervisor: L. J. Blok

1. PhD training	Year	Workload (Hours/ECTS)
General academic skills		
Laboratory animal science	2007	3 ECTS
Biomedical English Writing and Communication	2009-2010	4 ECTS
Research skills		
• Laser capture microdissection		10 hours
• Lentivirus making and RNAi		30 hours
• Confocal microscopy		10 hours
• Mouse ovariotomy, microsurgery and kidney capsule injection		20 hours
• Zebra fish maintaining and genes knockdown		10 hours
In-depth courses (e.g. Research school, Medical Training)		
• Certificate for radiation safety	2007	1 ECTS
• From development to disease	2007	1 ECTS
• Biomedical research techniques VI	2007	1 week
• English course 5A, Leiden	2007-2008	4.5 ECTS
• EndNote course	2008	4 hours
• Introduction of EDC	2008	4 hours
• 1st Erasmus Flow day	2009	8 hours
• InDesign course	2011	0.2 ECTS
Presentations and conferences		
• 11th Molecular Medicine Day, Rotterdam	2007	10 hours
• 1st "Stem Cells, Development and Regulation", Amsterdam	2007	16 hours
• Annual presentation at Josephine Nefkens Institute (JNI), Rotterdam (oral)	2008	40 hours
• 2nd "Stem Cells, Development and Regulation", Amsterdam	2008	10 hours
• Keystone meeting "Signalling Pathways in Cancer and Development", Steamboat Springs, Colorado, USA (poster)	2008	60 hours
• 12th Molecular Medicine Day, Rotterdam (poster)	2008	35 hours

• Wladmiroff onderzoeksprijs, Rotterdam (oral)	2009	40 hours
• The 2nd Dutch stem cells meeting, Rotterdam	2009	10 hours
• 13th Molecular Medicine Day, Rotterdam (oral)	2009	45 hours
• Leiden International Medical Student Conference, Leiden (oral)	2009	25 hours
• The AACR 101st Annual Meeting, Washington, DC, USA (poster, I didn't go due to the volcanic ash from Iceland)	2010	20 hours
• 14th Molecular Medicine Day (poster)	2010	35 hours
• Annual presentation at JNI, Rotterdam (oral)	2010	30 hours
• The 4th Dutch Stem Cell Meeting, Leiden, the Netherlands (oral)	2011	40 hours
• Masterclass with Rudolf Jaenisch, Amsterdam, the Netherlands (oral)	2011	30 hours
• Recent developments in stem cell research, Amsterdam, the Netherlands	2011	8 hours
• Erasmus Stem cell Institute (ESI) retreat, Nijverdal, the Netherlands	2011	2 days

Seminars and workshops

PhD day	2007	8 hours
Get out of lab days	2007	24 hours
Partek Data Analysis Workshop	2008	16 hours
5th workshop on innovative mouse models, Leiden	2009	16 hours
Weekly JNI meetings	2007-2011	
Monthly seminars from departments, institute and ESI	2007-2011	

2. Teaching activities

Supervising practicals and excursions

• Introduction of the lab and experimental skills	2008~2010	

Supervising Master's theses

• The binding proteins of progesterone receptor A and B	2009	4 months
• The characteristics of the uterine label-retaining cells	2009~2010	6 months

3. Grants

Trustfonds Erasmus University Rotterdam: travel grant	2008
Trustfonds Erasmus University Rotterdam: travel grant	2010

4. other academic activities

Reviewed articles from Journal of Molecular Medicine	2011
PhD lunch (talk to well-known scientists)	2007-2011

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LIST OF ABBREVIATIONS

Akt	protein kinase B
AMH	anti- Müllerian hormone
AMHR	AMH receptor
APC	adenomatosis polyposis coli
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CDH1	cadherin 1
CK1	casein kinase I
CK8	cytokeratine 8
DKK1	dickkopf 1
E2	estradiol
E9 (11.5 …)	embryonic day 9 (11.5 …)
ER	estrogen receptor
ESE	early secretory endometrium
EZH2	enhancer of zeste homolog 2
FCS	fetal calf serum
FOXO1	forkhead box protein O1
FZD	frizzled receptors
GSK3β	glycogen synthase kinase 3 beta
H2B-GFP	histone 2B - green fluorescent protein
hCG	human chorionic gonadotropin
HH	hedgehog
IGF	insulin-like growth factor
IHC	immunohistochemistry
IRS-1	insulin receptor substrate 1
LOH	loss of heterozygosity
LPL	lipoprotein lipase
LRCs	label retaining cells
LRP	low-density lipoprotein receptor related protein
LCM	laser capture microdissection
LSE	late secretory endometrium
LT-LRCs	long-term LRCs
MMP7	matrix metallopeptidase 7
MPA	medroxyprogesterone acetate
MSE	mid-secretory endometrium
OSEs	ovarian surface epithelial cells
PAEP	progestagen-associated endometrial protein
PE	proliferative endometrium
Pgr/PR	progesterone receptor
PI3K	phosphoinositide kinase-3
PIK3R2	PI3K regulatory subunit beta
Prg	progesterone
SAM	statistical analysis of microarray
SFRP2	frizzled-related protein 2
SMA	smooth muscle actin
TCF	T-cell factor
WISP2	Wnt1-induced signaling pathway protein 2

About the author

Yongyi Wang was born on September 15th, 1977 in Dalian, China. After graduating from the high school study at Jinzhou Senior Middle School, Dalian, he started his medical study at China Medical University. He received his Bachelor degree in Medicine in 2001 after he finished the clinical training at Red-Cross Hospital, Shenyang. In 2002, he continued his Master study of Biomedical Sciences at Leiden University and University of Amsterdam. During the master study, he performed two research projects with Dr. Jörg Hamann and Dr. Paula van Hennik at Academic Medical Centre Amsterdam. Immediately after his master graduation, he started with a PhD position at University of Basel, Switzerland. Later he recognized that he would like to do something different in the future. In 2007, he moved back to the Netherlands to pursue current PhD project at the Department of Obstetrics & Gynaecology, Erasmus University Medical Center, Rotterdam, under the supervision of Dr. LJ Blok (Dep. of Obstetrics & Gynaecology), Prof. Dr. JA Grootegoed (Dep. of Reproduction and Development) and Prof. Dr. R Fodde(Dep. of Pathology). His PhD projects involved the identification of stem cells and signaling pathways in uterus and fallopian tubes. Currently, he is working as a post-doc in the Department of Hematology, Erasmus University Medical Center.

Signaling pathways and stem cells in uterus and fallopian tubes

Yongyi Wang