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Lora Hedrick Ellenson *Editor*

Molecular Genetics of Endometrial Carcinoma

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Preface

Nearly every seminar, discussion and resident or medical school lecture, begins with the fact that endometrial carcinoma is the most common malignancy of the female genital tract in the United States. Despite this, our fundamental understanding of the disease entities that make up this category of carcinoma has remained shallow. Consequently, the treatment and survival of women with these diseases has remained relatively unchanged over the past four decades. The convergence of information coming from molecular geneticists, signal transduction biologists, epidemiologists, and gynecological pathologists and oncologists, however, has led to a rapid deepening of our understanding of endometrial carcinoma. This text, written by many leaders in the field, has been prepared in response to the sheer volume of information that has been produced over the last approximately 5 years. Surely, by the time it gets to press, there will be additional new information on endometrial carcinoma. But the goal of the text is to serve as a resource for investigators and clinicians to understand what has been done and what needs to be done to decrease the morbidity and mortality of women with endometrial carcinoma.

New York, NY, USA — Lora Hedrick Ellenson

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Part I
Introduction

Chapter 1
Epidemiology of Endometrial Carcinoma: Etiologic Importance of Hormonal and Metabolic Influences

Ashley S. Felix, Hannah P. Yang, Daphne W. Bell, and Mark E. Sherman

Abstract Endometrial carcinoma is the most common gynecologic cancer in developed nations, and the annual incidence is projected to increase, secondary to the high prevalence of obesity, a strong endometrial carcinoma risk factor. Although endometrial carcinomas are etiologically, biologically, and clinically diverse, hormonal and metabolic mechanisms are particularly strongly implicated in the pathogenesis of endometrioid carcinoma, the numerically predominant subtype. The centrality of hormonal and metabolic disturbances in the pathogenesis of endometrial carcinoma, combined with its slow development from well-characterized precursors in most cases, offers a substantial opportunity to reduce endometrial carcinoma mortality through early detection, lifestyle modification, and chemoprevention. In this chapter, we review the epidemiology of endometrial carcinoma, emphasizing theories that link risk factors for these tumors to hormonal and metabolic mechanisms. Future translational research opportunities related to prevention are discussed.

Keywords Endometrial carcinoma • Incidence trend • Risk factors • Estrogen • Progesterone • Hormones • Insulin • Inflammation • Adipokines

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Descriptive Epidemiology

Endometrial carcinomas develop from the inner lining of the uterine corpus and account for the substantial majority of tumors affecting the organ [1]. Accordingly, descriptive epidemiological data for uterine cancer, which is frequently the best available category in cancer registries, are often used as a surrogate for endometrial carcinoma rates, as presented below.

Worldwide, there are an estimated 319,500 incident uterine cancers reportedly annually, which account for over 76,000 deaths each year [2]. Incidence rates vary widely; age-standardized incidence rates are higher in North America and most of Europe than in other parts of the world (Fig. 1.1). Within the United States, uterine cancer incidence rates peaked around 1975 in relation to increased use of exogenous unopposed estrogens [3, 4] (Fig. 1.2). After recognition that the use of unopposed estrogens is carcinogenic in the endometrium, the use of these products declined, and age-adjusted endometrial carcinoma incidence rates fell in parallel and then leveled from 1988 to 2006. Subsequently, from 2006 to 2011, incidence rates increased by 2.3 % per year.

In 2015, uterine cancer is estimated to be the fourth most common cancer diagnosed among American women, only exceeded by the incidence of cancers of the breast, lung and bronchus, and colon and rectum [5]. It is estimated that there will be approximately 54,870 new cases of uterine cancer in the United States in 2015 [5]. Studies have projected that uterine cancer incidence rates will continue to rise over the next 15 years [6, 7]. Given increases in the US total population and the rising proportion of older women, these projections suggest an important increase in the uterine cancer burden.

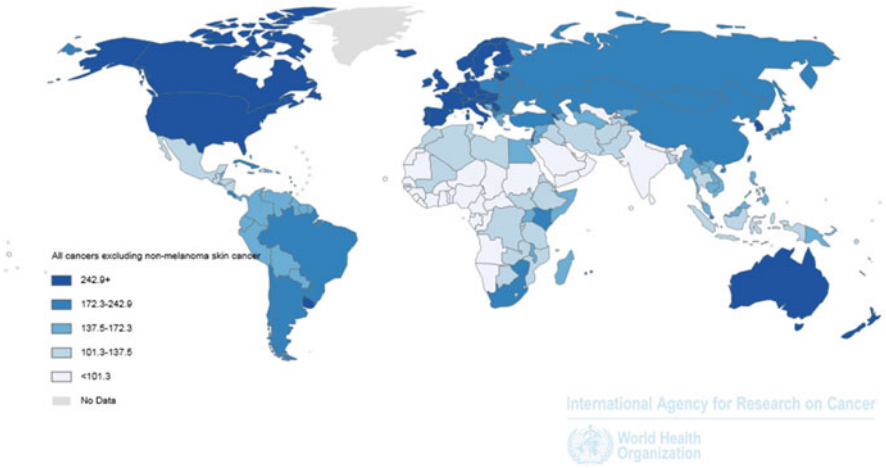

Fig. 1.1 International incidence for uterine cancer (per 100,000 woman years) age standardized to the world population, 2012 (*Source*: GLOBOCAN 2012 (IARC))

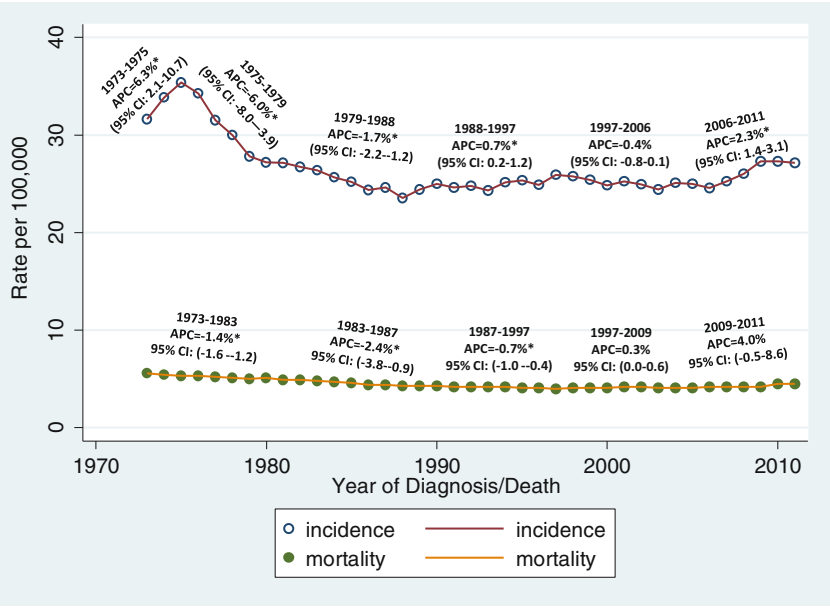

Fig. 1.2 Trends in uterine cancer incidence and mortality rates in the United States 1973–2011

Uterine cancer is most commonly diagnosed after menopause, with the peak incidence occurring between ages of 60 and 70 years. Reported uterine cancer incidence rates among White women have consistently been higher than among non-White women in the United States, but this interpretation is limited by failure to correct for hysterectomy prevalence in registry data (see below) [1]. Between 1999 and 2008, reported incidence rates were relatively stable among White women (average annual percent change = 0.1 %), but increased among Black women (1.8 %), Asian–Pacific Islanders (1.9 %), and Hispanics (1.2 %) [8]. Registry data also show that age-adjusted incidence rate trends differ by histologic tumor subtype (*discussed below*), with increased incidence of lower-grade endometrioid carcinomas during 1999 through 2006 compared with other subtypes, which remained relatively stable during the same period [9].

Uterine cancer accounts for about 2 % of cancer deaths among women in high-income nations [2]. Age-standardized uterine cancer mortality rates are highest in parts of the Caribbean (3.3 per 100,000), Central and Eastern Europe (3.4), Melanesia (3.8), and Micronesia/Polynesia (2.5) and lower in the United States (2.2) [2]. Uterine cancer mortality rates among American women have decreased over the past few decades and have been relatively stable from 1997 to 2009, with a slight rise after 2009 [10] (Fig. 1.2). It is estimated that there will be approximately 10,170 deaths related to uterine cancer in the United States in 2015 [5]. Although Black women experience a lower reported incidence of endometrial carcinoma, they are more than twice as likely to die from the disease as White women [8]. Registry data demonstrate increasing mortality rates in Asian–Pacific Islanders (average annual percent change = 1.9 %) and non-Hispanics (0.3 %) and steady rates in Whites (0.1 %), Blacks (0.5 %), and Hispanics (0.0 %) from 1999 to 2008 [8].

Reported incidence and mortality rates are not corrected for hysterectomy prevalence and, therefore, underestimate rates among women who are at risk but have not undergone a hysterectomy [11, 12]. Hysterectomy prevalence may vary by race and likely other factors; thus, incidence rate ratios that are not corrected for hysterectomy prevalence may be misleading. Further, imperfect distinction of endocervical from endometrial adenocarcinoma, especially prior to routine use of diagnostic immunohistochemical markers, represents another source of error, particularly in older datasets.

Trend analysis based on hysterectomy-corrected data from 1992 to 2008 showed that the endometrial carcinoma incidence rate significantly declined 0.8% per year among White women compared to an increase rate of 3.1% per year among Black women, such that the incidence rates for Black women surpassed those among White women from 2004 to 2008 [12]. Hysterectomy-corrected incidence rates increased for all major histopathologic subtypes among Black women, but declined or showed statistically nonsignificant increases among White women. Another analysis reported that hysterectomy correction had the largest effect on incidence in the southern states in the United States, where hysterectomy prevalence was highest irrespective of race [11].

Most endometrial carcinomas present clinically with abnormal uterine bleeding and vaginal discharge, leading to diagnosis at an early stage [13]. Based on recent SEER 18 data (2004–2011), the estimated overall 5-year survival rate for uterine cancer is 81.5% [14] (Table 1.1). However, prognosis is less favorable among women with non-endometrioid carcinomas and tumors that are higher grade and higher stage (Table 1.1). The current standard management of endometrial carcinoma is total hysterectomy, bilateral salpingo-oophorectomy, and pelvic and para-aortic lymphadenectomy [13]. Women with advanced pathologic stage may receive adjuvant therapy, including radiation, vaginal brachytherapy, and chemotherapy [15].

Table 1.1 Five-year survival proportions of uterine cancer by histology and stage in the United States 2004–2011

	All stages (%)	Localized (%)	Regional (%)	Distant (%)
All uterine cancer cases	81.5	95.2	68.2	25.0
Endometrioid	91.5	97.6	79.9	43.4
Mucinous	91.8	98.7	79.2	9.6
Adenocarcinoma	81.1	95.9	63.9	14.7
Clear cell	60.2	86.8	58.3	23.2
Serous	48.4	82.1	47.6	17.0

Actuarial method. Ederer II method used for cumulative expected

Source: Surveillance, Epidemiology, and End Results (SEER) Program (www.seer.cancer.gov) SEER*Stat Database: Incidence—SEER 18 Regs Research Data + Hurricane Katrina Impacted Louisiana Cases, Nov 2013 Sub (1973–2011 varying)—Linked To County Attributes—Total U.S., 1969–2012 Counties, National Cancer Institute, DCCPS, Surveillance Research Program, Surveillance Systems Branch, released April 2014 (updated 5/7/2014), based on the November 2013 submission

Classification

Classification of endometrial carcinomas based on etiological factors, histopathologic type, or molecular markers demonstrates substantial, although imperfect consistency. Future development of taxonomies that integrate patient and tumor characteristics may ultimately result in more homogeneous biological categories.

Bokhman's seminal paper in 1983 describing two main types of endometrial carcinomas, based mainly on clinical presentation, laid the framework for developing refined taxonomies that integrate patient and tumor features [16]. As originally described [16], type I carcinomas comprised about 80 % of cancers and were associated with signs and symptoms linked to endocrine and metabolic disturbances. Type I tumors overlap considerably with cancers histopathologically classified as endometrioid, and particularly those that are low or intermediate tumor grade, superficially invasive into the myometrium, and low stage. Type II carcinomas affect women with less overt evidence of hormonal or metabolic dysfunction and, pathologically, tend to be high grade, deeply invasive into the myometrium, and higher stage at detection. Serous carcinomas are perhaps the best histopathologic correlate of type II carcinomas. The dichotomous division of endometrial carcinomas into two (but potentially more histopathologic subtypes) has been modified over time and expanded using modern molecular biology techniques.

The Tumor Cancer Genome Atlas (TCGA) provides a molecular taxonomy of endometrial carcinoma based on integrated multi-platform genomic profiling [17]. Molecular stratification of 248 tumors according to somatic copy-number status, somatic mutation status, and microsatellite instability (MSI) status led to the description of four main molecular subgroups: (1) ultramutated/polymerase E (*POLE* (*polymerase (DNA directed)*, *epsilon*, *catalytic subunit*)) mutant, (2) hypermutated/microsatellite unstable, (3) copy-number low/microsatellite stable, and (4) copy-number high/serous-like. The first three subgroups are composed largely of endometrioid carcinomas, which approximate Bokhman's type I tumors. In contrast, 94 % of serous carcinomas, 24 % of high-grade endometrioid carcinomas, and 62 % of carcinomas of mixed histologic type are included in the copy-number high/serous-like subgroup. Somatic mutations that are frequent among tumors in subgroups 1–3 correspond to those associated with endometrioid carcinoma overall: *PTEN* (*phosphatase and tensin homolog* (and other members of *PI(3)kinase/AKT* pathway)), *FGFR2* (*fibroblast growth factor receptor*), *ARID1A* (*AT-rich interactive domain 1A* (*SWI-like*)), *CTNNB1* (*catenin* (*cadherin-associated protein*), *beta 1*, 88 kDa), and *KRAS* (*Kirsten rat sarcoma viral oncogene homolog*). Subgroup 2 is associated with high rates of MSI, mostly reflecting DNA promoter methylation silencing of *MLH1* (mutL homolog 1). Mutations found in subgroups 1–3 are much rarer among copy-number high/serous-like tumors, which in contrast, are associated with *TP53* (*tumor protein P53*) mutations in 90 % of cases and frequent copy-number alterations, neither of which are prominent features of subgroups 1–3.

Overall, the genomic profile of copy-number high/serous-like endometrial tumors show similarities with basal breast cancers and serous "ovarian" (which

likely includes many fallopian tube primaries) carcinomas, which are also clinically aggressive and less strongly linked to hormonal etiology as compared with other tumor subtypes occurring in their respective sites of origin. Among *TP53*-mutant endometrioid carcinomas (i.e., high grade) in the copy-number high/serous-like subgroup, 50 % had concurrent *PTEN* mutations as compared with only 2.6 % of serous carcinomas in this subgroup, suggesting that the pathogenesis of carcinomas in the copy-number high/serous-like group may itself be diverse.

It is unclear whether *TP53* mutations represent an early event in the pathogenesis of a subset of endometrioid carcinomas (possibly implying a distinctive etiology) or a late event occurring in established endometrioid carcinomas (reflecting clonal evolution). These possibilities may underscore inconsistencies in etiological associations. For example, in some analyses, risk factor associations for grade 3 endometrioid carcinomas are more similar to non-endometrioid carcinomas than to grade 1 or 2 endometrioid carcinomas [18]. This could reflect the development of a subset of grade 3 endometrioid carcinomas via a "subgroup 4" nonhormonal pathway. In addition, given that distinguishing serous carcinomas from high-grade endometrioid carcinomas [19] is often difficult, misclassification of endometrioid carcinomas that developed via hormonal mechanisms as serous carcinomas could blur etiological distinctions between these subtypes. In fact, some tumors that initially develop as hormonally driven low-grade endometrioid carcinomas may progress to mixed tumors in which the serous component overgrows and obscures the endometrioid areas. This hypothetical scenario could result in serous carcinomas that ostensibly are associated with hormonal risk factors.

TCGA RNA sequencing data suggests that there are three endometrial carcinoma transcriptome clusters: "hormonal," "mitotic," and "immunoreactive" [17]. Within the hormonal transcriptome cluster, the levels of *ESR1* (*estrogen receptor 1*) and *PGR* (*progesterone receptor*) mRNA expression, and the levels of estrogen receptor (ER) and progesterone receptor (PR) protein expression, are significantly higher than in either the mitotic or immunoreactive tumor clusters. Moreover, increased levels of progesterone receptor (PR) expression are also characteristic of tumors in the copy-number low/microsatellite stable molecular subgroup, similar to the hormonal transcriptome cluster. Given that excess exposure to estrogen relative to progesterone is proposed as an important mechanism in endometrial carcinogenesis, the identification of tumors with high PR expression may identify tumors that demonstrate distinct associations with hormonal exposures and relative susceptibility to endocrine chemopreventive and treatment strategies.

A subsequent analysis of the TCGA transcriptome and reverse-phase protein array data focused exclusively on endometrioid carcinomas and described four, rather than three, expression clusters [20]. In this classification, one of the four clusters (cluster I) exhibited high expression of *ESR* and *PGR*, was statistically significantly enriched for microsatellite unstable tumors, and was composed almost exclusively of *PTEN*-mutated tumors [20]. Notably, a second cluster (cluster II) was associated with younger obese patients, high rates of *CTNNB1* mutations, and lower survival than patients in cluster I. Since both clusters I and II are associated with obesity, their underlying molecular heterogeneity has been suggested as a possible explanation for some of the clinical heterogeneity that is seen among patients with endometrioid endometrial carcinoma [20].

Considering gene expression data together with copy-number data and pathway interaction data, TCGA has also described five so-called "PARADIGM" tumor clusters, one of which (PARADIGM cluster 5) is enriched with cases from the hormonal expression cluster and shows high levels of MYC (v-myc avian myelocytomatosis viral oncogene homolog), FOXA1 (forkhead box A1), and HIF1 (hypoxia-inducible factor 1), alpha subunit (basic helix-loop-helix transcription factor) signaling [17]. This observation is consistent with the biochemical observations that the *c-myc* proto-oncogene is transcriptionally regulated by estrogen [21], the FOXA1 transcription factor can modulate the estrogenic response in breast cancer cells by facilitating binding of ER to target sites on chromatin [22], and *HIF1A* mRNA and protein levels increase in the rat uterus upon estradiol stimulation [23]. Furthermore, the association between ostensibly "hormonally driven" endometrial carcinomas and elevated MYC, FOXA1, and HIF1 signaling noted by TCGA is largely consistent with the findings of other studies of endometrioid carcinomas. For instance, a positive correlation ($r=0.37$, $p=0.038$) has been noted between ERα and c-myc protein expression by immunohistochemical staining in a series of predominantly endometrioid endometrial carcinomas [24]. Likewise, a positive association between ER levels and FOXA1 levels has been noted in primary endometrial carcinomas [25, 26], and a trend toward such an association has been suggested in another study [27]. Moreover, low FOXA1 expression in primary endometrial carcinomas shows a significant association with non-endometrioid histology ($P=0.002$), high tumor grade ($P=0.003$), PR loss ($P=0.02$), ERα loss ($p=0.003$), and reduced disease-specific survival ($p=0.004$) [26]. Finally, a trend toward an association between HIF-1α, HIF-2α, and ER expression has been reported in endometrial carcinomas, but these associations were only of borderline statistical significance ($P=0.06$) [28].

Imbalances in Estrogen and Progesterone as the Main Driver of Endometrial Carcinogenesis

Imbalances in sex steroid hormones—excess stimulation of endometrial epithelium by estrogen relative to progesterone—are often conceptualized as a leading paradigm to account for the etiology of endometrial carcinomas (i.e., mainly type I) [29]. Estrogen, when insufficiently opposed by progesterone, has proliferative effects on the endometrium, which may result in a higher probability of random mutations in oncogenes and tumor suppressor genes. Endometrial cells that acquire multiple mutations without appropriate repair mechanisms may gain a growth advantage and develop into clones of cancer cells [30].

This overarching framework is supported by several lines of compelling evidence. First, in healthy premenopausal women, endometrial cell division rates are highest during the proliferative phase of the menstrual cycle, when estradiol levels are high and progesterone levels are low [31]. However, it is postulated that among premenopausal women, physiological levels of estrogen drive maximal proliferation, suggesting that progesterone deficiency, DNA repair defects, or other factors may

figure importantly in the early pathogenesis of endometrial carcinomas [32]. During the secretory phase of the menstrual cycle, a surge in progesterone levels is followed by a plateau of endometrial cell division, secretory differentiation, and then apoptosis prior to menstrual shedding.

In addition, three prospective studies [33–35], which included between 124 and 250 postmenopausal endometrial carcinoma cases, reported positive associations between higher circulating estradiol levels and endometrial carcinoma risk. The relative risk (RR) comparing the highest vs. lowest category of estradiol ranged from 2.1 (95% confidence interval (CI) = 1.2–3.6) [34] to 4.1 (95% CI = 1.8–9.7) [33]. Further, studies that assess endometrial carcinoma risk in relation to circulating levels of sex hormone-binding globulin (SHBG), a protein that binds to estrogen, thereby lowering its bioavailable fraction, report low levels of SHBG which are related to higher endometrial carcinoma risk [33, 34].

Finally, epidemiologic studies (*reviewed in next section*) have shown that factors related to greater lifetime exposure to sex steroid hormones, and more specifically estrogens, including younger age at menarche, older age at menopause, postmenopausal use of unopposed estrogen, and high postmenopausal body mass index (BMI), are associated with increased risk of developing endometrial carcinoma. Conversely, factors related to lower lifetime exposure to estrogen relative to progesterone, such as parity, postmenopausal use of estrogen plus progestin, and combined oral contraceptive (COC) use are related to lower endometrial carcinoma risk.

Estrogen and Progesterone Receptors in Endometrial Tumor Tissues

Evaluating ER and PR expression and function in endometrial tissues is complex. Given that the functionalis (superficial) endometrium is shed cyclically, it is supposed that the deeper basalis, which is not shed with menses, may be the site where stem/progenitor cells reside; accordingly, expression of ER and PR in the basalis may be important in understanding carcinogenesis, but this compartment is only accessible for study in hysterectomy samples, precluding longitudinal study. However, and perhaps paradoxically, the basalis is generally viewed as less hormonally responsive than the functionalis. Further, expression of ER and PR varies across the menstrual cycle and reproductive life, suggesting both temporal and spatial heterogeneity. There are two major forms of each hormonal receptor (ERα, ERβ; PRA, PRB), which may have different functions, and ERβ has multiple splice variants with potentially distinctive actions. Immunohistochemistry has important utility in investigations of hormone receptors in endometrial research because of the intermixing of multiple cell types in normal tissue and the variation in cellular composition over the menstrual cycle and the life course. Accordingly, molecular profiling of tissues without dissection may be difficult to interpret because of cellular admixtures. However, the sensitivity and specificity of reagent antibodies for various hormone markers,

particularly ERβ in older studies, have been questioned [36]. Finally, important physiological differences between mice and women raise questions about the relevance of hormonal studies in mice and their relevance to women.

Estrogen exerts its cellular effects via its interaction with the ERα, ERβ, or GPER (G protein-coupled estrogen receptor 1) receptors. Studies of ERα, ERβ, and ERα/β knockout mice have pointed to ERα as the primary mediator of the proliferative response to estrogen in the endometrium and as a transcriptional activator of the progesterone receptor gene in endometrial stromal cells [37]. ERβ can mediate an antiproliferative response by antagonizing the effects of ERα in the endometrium [38], but the role of ERβ in endometrial carcinogenesis is unclear and may differ from effects at other organ sites [39], whereas ERα dysregulation in endometrial carcinoma has been studied extensively.

PR is expressed in both the epithelial and stromal cells of the endometrium, and stromal PR acts in a paracrine manner to inhibit proliferation of the glandular epithelial cells [40]. PRA and PRB constitute the two major isoforms of the progesterone receptor, with PRA being the predominant isoform in endometrial stromal cells [41]. Progesterone can mediate distinct biochemical and cellular responses via its interaction with PRA or PRB (reviewed in [42]).

In endometrial tumor tissues and preoperative curettage specimens, both ER and PR protein expression status are closely correlated with tumor histology, grade, depth of myometrial invasion, and clinical outcome [43–53]. Positivity for ER, ERα, PR, PRA, and PRB expression is observed more often in low-grade than high-grade tumors [43, 46–49, 54] and in endometrioid than non-endometrioid carcinomas [44, 48], which is consistent with unopposed estrogen being a strong epidemiological risk factor for the endometrioid subtype. Loss of ER and PR expression is significantly associated with deep myometrial invasion [43, 44, 46]. In both endometrioid and non-endometrioid subtypes, metastatic tumors demonstrate loss of PR expression more often than primary tumors [48]. Within the endometrioid subtype, loss of ER and PR expression correlates with increasing tumor grade and stage [48, 50].

A considerable body of work supports ER, PR, or joint ER/PR protein expression status, as independent prognostic indicators of clinical outcome for endometrial carcinoma [45, 47, 48, 55–58]. Concurrent ER/PR loss is an independent predictor of tumor recurrence [47], lymph node metastasis [56], and reduced disease-specific survival [56]. In early stage disease, losses of ER and ERα protein expression are, respectively, independent predictors of recurrence and death from disease [47, 50]. PR expression status of endometrial carcinomas has been found to be an independent prognostic indicator in several studies (reviewed in [59]). Moreover, loss of PRA expression in early stage disease has been reported to be an independent prognostic factor for relapse [50].

Loss of PR expression in endometrial tumor tissues may also be accompanied by an underlying change in the ratio of PRA and PRB expression [53, 60]. Loss of PRA or PRB expression has been noted in 51–75% of endometrioid endometrial cancers [53, 61], with some studies noting PRB loss more often than PRA loss [53, 60], and others finding the converse [51, 61]. The fraction of endometrioid carcinomas positive

for PRA and PRB expressions declines with increasing tumor grade [61]. Although the expression of PRA and PRB is lower in endometrioid carcinomas than in endometrial hyperplasia (specifically complex atypical hyperplasia (CAH)), data conflict as to whether the balance between PRA and PRB expression is related to functional dysregulation in these early lesions [51, 53]. In one study, a univariate analysis reported that a PRA:PRB ratio of <1 was associated with shorter disease-free survival and disease-specific death [50].

Although ER and PR status are not routinely used in clinical decision-making for endometrial carcinoma, recent reviews have suggested they might be incorporated into clinical practice as biomarkers for risk stratification [62, 63].

Estrogen and Progesterone Tissue Levels

Bernstein et al. [64] noted that endometrial tumor tissues exhibit higher concentrations of estradiol compared with normal endometrial tissue. Among 78 adenocarcinomas, estrogen levels were higher in low-grade tumors versus high-grade tumors, and in more-invasive tumors versus less-invasive tumors, although these associations did not achieve statistical significance.

Dysregulated expression, in endometrial tumor tissues, of genes and proteins modulating estrogen biosynthesis and metabolism has been the topic of a number of investigations, although findings are not entirely consistent (reviewed in [65]). Estrogen biosynthesis in peripheral tissues, including the endometrium, is driven by the aromatase and sulfatase pathways. The conversion of estrone sulfate to estrone is catalyzed by STS (steroid sulfatase (microsomal), isozyme S) and antagonized by SULT1E1 (sulfotransferase family 1E, estrogen-preferring, member 1) and SULT1E2 (sulfotransferase family 1E, estrogen-preferring, member 1). Compared to normal endometrium, the ratio of *STS*:*SULT1E1* mRNA and protein expression is increased in endometrial tumor tissues (reviewed in [65]). STS also promotes the conversion of DHEA-sulfate to DHEA, an effect that is antagonized by the actions of SULT2A1 (sulfotransferase family, cytosolic, 2A, dehydroepiandrosterone (DHEA)-preferring, member 1) and SULT2B1 (sulfotransferase family, cytosolic, 2B, member 1). *SULT2B1* expression is increased in tumor versus adjacent normal endometrium, and moderate levels of the SULT2B1 are detectable in endometrial tumors by immunohistochemistry [65, 66].

CYP19A1 (*cytochrome P450*, *family 19*, *subfamily A*, *polypeptide 1*) mRNA levels are low in endometrial tumor tissue and are similar to that of adjacent normal tissue [65, 67]. In contrast CYP19A1 (aromatase) protein expression, as assessed immunohistochemically, varies widely, possibly related to methodological differences between studies [50, 65, 68–70].

Expression of *HSD3B1* (*hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1*) and *HSD3B2* (*hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2*), the products of which promote the conversion

of DHEA to androstenedione, appears similar in endometrial tumors versus adjacent normal endometrium [71]. AKR1C3 (aldo-keto reductase family 1, member C3) promotes the conversion of androstenedione to testosterone, whereas HSD17B2 (hydroxysteroid (17-beta) dehydrogenase 2) converts testosterone to androstenedione. Whereas *AKR1C3* (aldo-keto reductase family 1, member C3) expression appears similar between adjacent tumor and normal tissues by real-time PCR [71–73], microarray data generated within TCGA indicate increased expression of *AKR1C3* in 6% of high-grade endometrial tumors [17, 74].

HSD17B1 (hydroxysteroid (17-beta) dehydrogenase 1), HSD17B7 (hydroxysteroid (17-beta) dehydrogenase 7), and HSD17B12 (hydroxysteroid (17-beta) dehydrogenase 12) catalyze the conversion of estrone to estradiol. Their expression appears to be unchanged in endometrial carcinomas compared with normal endometrium, and most endometrial carcinomas show weak immunohistochemical staining for these three proteins [65, 73, 75, 76]. The reverse reaction (conversion of estradiol to estrone) is catalyzed by HSD17B2 (hydroxysteroid (17-beta) dehydrogenase 2) as well as HSD17B4 (hydroxysteroid (17-beta) dehydrogenase 4) and HSD17B8 (hydroxysteroid (17-beta) dehydrogenase 8). Several studies have noted increased expression of *HSD17B2* in endometrial tumors, and the HSD17B2 protein is detectable in endometrial carcinomas by IHC [65, 71, 76, 77]. *HSD17B4* and *HSD17B8* expressions do not appear to differ between endometrial tumor and normal tissues, and tumors show moderate immunohistochemical staining [65, 73].

The expression of genes that regulate estrogen metabolism has also been evaluated in endometrial tumor tissues (reviewed in [65]). As compared with normal adjacent endometrium, endometrial tumors have been observed to have decreased *CYP1B1* (*cytochrome P450, family 1, subfamily B, polypeptide 1*) expression; increased or unchanged *CYP1A7* expression; decreased *CYP3A7* (*cytochrome P450, family 3, subfamily A, polypeptide 7*) expression; unchanged *CYP3A5* (*cytochrome P450, family 3, subfamily A, polypeptide 5*) expression; increased or unchanged *COMT* (*catechol-O-methyltransferase*) expression; increased *UGT2B7 (UDP glucuronosyltransferase 2 family, polypeptide B7)*, *UGT2B15 (UDP glucuronosyltransferase 2 family, polypeptide B15)*, *UGT1A1 (UDP glucuronosyltransferase 1 family, polypeptide A1)*, and *UGT1A3* (*UDP glucuronosyltransferase 1 family, polypeptide A3*) expressions; and increased or unchanged expression of *GSTP1* (*glutathione S-transferase Pi 1*) expression [65, 66, 76, 78, 79].

The expression of genes associated with local progesterone biosynthesis and metabolism has recently been compared between endometrial carcinoma tissues and adjacent normal endometrial tissue [71]. The local biosynthesis of progesterone from cholesterol is dependent on the activities of STAR (steroidogenic acute regulatory protein), CYP11A1 (cytochrome P450, family 11, subfamily A, polypeptide 1), HSD3B1 (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1), and HSD3B2 (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2). *STAR* and *CYP11A1* expressions are decreased in endometrial tumor tissues, whereas *HSD3B1* and *HSD3B2* expressions appear to be unchanged [71]. Progesterone is metabolized by the concerted actions of

AKR1C1 (aldo-keto reductase family 1, member C1), AKR1C2 (aldo-keto reductase family 1, member C2), AKR1C3 (aldo-keto reductase family 1, member C3), SRD5A1 (steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)), and SRD5A2 (steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2)), and metabolism can be antagonized by the activity of HSD17B2 (hydroxysteroid (17-beta) dehydrogenase 2). *HSD17B2* expression and *SRD5A2* expression are increased in endometrial tumor tissues, whereas *SRD5A2* expression is unchanged [71]. Data on *AKR1C1–3* gene expression in endometrial tumors is variable. Whereas several studies found no change in *AKR1C1*, *AKR1C2*, and *AKR1C3* expressions between adjacent tumor and normal tissues using real-time PCR [71–73, 77], microarray data generated within TCGA indicate increased expression of *AKR1C1*, *AKR1C2*, and *AKR1C3* in 4–6 % of high-grade tumors [17, 74], and immunohistochemical analysis of AKR1C3 indicates both increased and decreased expression in endometrial carcinoma compared with endometrial hyperplasia [80, 81]. These variable findings might reflect differences in study design or inter-patient variability as suggested by Rižner and Penning [74].

ESR1 and PGR Mutations in Endometrial Carcinoma

ESR1, which encodes ERα, is somatically mutated in about 4 % of endometrial carcinomas, and the mutations described thus far localize to the ligand-binding domain or to the DNA-binding domain of ERα [17, 82]. Within the ligand-binding domain, codons 547 and 548 are recurrently mutated in endometrial carcinomas and encode constitutively active, gain-of-function mutants ($ESR1^{Y537S/C/N}$ and $ESR1^{D538G}$) [82, 83]. Because *ESR1*-mutated breast tumors are associated with prior treatment with antiestrogens and aromatase inhibitors, it has been speculated that *ESR1*-mutated endometrial carcinomas may be associated with tamoxifen treatment for concurrent breast cancer [82], although this hypothesis remains to be tested. The frequency of *ESR1* gene amplification in endometrial carcinomas exhibits considerable inter-study variability, with amplification noted in 1–23 % of tumors [17, 84, 85], likely reflecting differences in methodological approaches used to assess copy number and possibly population differences [86]. In the TCGA cohort, 6.7 % of 240 endometrial carcinomas have somatically mutated or deleted *PGR* [17, 87].

In summary, available data do not provide an entirely clear picture of hormone metabolism at the endometrial tissue level. However, the strong links between hormonal risk factors, exogenous hormone use, and serum hormone levels with endometrial cancer risk underscore the importance of systemic hormone imbalances in endometrial cancer etiology.

Mouse Models

Contributions of Estrogen and Progesterone to Endometrial Tumorigenesis in PTEN Knockout Mouse Models

PTEN tumor suppressor gene abnormalities are frequently identified in endometrioid carcinomas and its precursors [88–91], and focal loss of immunohistochemical expression in normal-appearing endometrial glands has been found in 20–40 % of benign endometrium ([92] and unpublished). Moreover, women with Cowden syndrome, which is related to germline *PTEN* mutations, are at increased risk of developing endometrial carcinoma, providing further support for the importance of *PTEN* perturbations in endometrial tumorigenesis [93, 94]. However, *PTEN* mutations alone are insufficient to initiate endometrial carcinoma since approximately 20–40 % of women have normal-appearing endometria that demonstrate small foci of PTEN-null glands, whereas the lifetime risk of endometrial carcinoma is approximately ten times lower [92]. Additional events that are believed to cooperate with *PTEN* loss to promote endometrial carcinoma include perturbations in other genes, as well as hormonal influences [92, 95]. In regard to the latter point, because unopposed estrogen is a well-established risk factor for endometrioid endometrial carcinoma, there has been great interest in understanding the interplay between steroid hormones and *Pten* loss in the development of endometrial carcinoma, using mouse models.

Studies in oophorectomized *Pten*+/− mice, and in *Pten*+/−/*ERα*-/− mice, have shown that the development of CAH and endometrial adenocarcinoma is independent of estrogen, although estrogen appears to potentiate the outgrowth of invasive carcinoma [96–98]. Similar findings have been made in a mouse model ($Pten^{loxP/loxP}$) with conditional deletion of *Pten* in the uterus, in which development of CAH and endometrial carcinoma is also independent of estrogen [99, 100]. Mechanistically, the development of hyperplasia in the absence of estrogen may be explained by the fact that loss of Pten function leads to Akt-dependent phosphorylation on ERα-Ser167, resulting in ligand-independent activation of ERα [98]. These observations may be relevant to human endometrial carcinomas since the estrogen independence of CAH in mouse models provides a rationale for the fact that, clinically, some patients present with hyperplasia in the absence of discernible clinical signs of hyperestrogenism [96].

The effect of progesterone on endometrial tumorigenesis in *Pten* mouse models has also been investigated. In oophorectomized *Pten*+/− mice, pretreatment with medroxyprogesterone acetate is insufficient to prevent the development of hyperplasia and adenocarcinoma [97]. Likewise, in oophorectomized mice ($PR^{cre/+}$ $Pten^{f/f}$) with conditional deletion of *Pten* in the uterus, progestin pretreatment is unable to prevent endometrial tumor progression, and tumors arising in this context have increased PR expression in the stroma [99]. Furthermore, progesterone alone is insufficient to cause endometrial tumor regression in an endometrial regeneration model in which *Pten*-ablated epithelial cells are admixed with *Pten*-wild type stromal cells [101]. However, in this same regeneration model, co-treatment with progesterone and estrogen results

in endometrial tumor regression, and this effect is dependent on intact PR expression in the stromal cells [101]. Moreover, when mutant KRAS (G12D) is introduced into the *Pten*-ablated epithelial cells in the regeneration model, the outgrowing tumors exhibit reduced stromal PR levels, similar to observations in $Pten^{d/d}Kras^{G12D}$ uteri [102] and are refractory to progesterone and estrogen co-treatment, an effect that is reversed by the overexpression of exogenous PR in the stromal cells [101].

Obesity and Endometrial Carcinoma in Animal Models

The obese Zucker (*fa*/*fa*) rat serves as an animal model for metabolic syndrome [103]. In terms of their response to estrogen exposure, the endometrium of oophorectomized Zucker rats treated with 17β-estradiol exhibits increased expression of proliferative markers (cyclin A and c-myc), decreased expression of antiproliferative markers (p27Kip1 and sFRP4), and increased Erk1/Erk2 activation, as compared with the endometrium of lean controls [104].

Risk Factors for Endometrial Carcinoma

The epidemiologic evidence implicates factors that increase a woman's exposure to circulating estrogen, relative to progesterone, as the main etiologic drivers of endometrial carcinoma risk (Table 1.2). In this section, we describe relationships between risk factors that are established in the etiology of endometrioid endometrial carcinomas, the most common histologic subtype, with a focus on factors hypothesized to act via hormonal mechanisms. Conceptually, exposures mediated by hormones might act through one or more mechanisms: (1) greater cumulative exposure to estrogens over a lifetime; (2) exposure to supraphysiologic estrogen levels, given the phase of a woman's life course (e.g., postmenopausal levels are physiologically low); and (3) progesterone deficiency (Fig. 1.3).

Non-contraceptive Postmenopausal Hormone Use

Endometrial carcinoma has long been recognized as a hormonally responsive tumor [3]. As mentioned in an earlier section, the introduction of unopposed estrogen therapy for amelioration of menopausal symptoms was followed by a dramatic increase in the incidence of endometrial carcinoma in the United States [105, 106]. Based on 29 epidemiologic studies, Grady and colleagues [107] reported an RR of 2.3 [95% CI = 2.1–2.5] associated with ever use of unopposed estrogen therapy compared with never use. The increased risk became apparent after 1–5 years of use [RR (95% CI) = 2.8 (2.3–3.5)], with an increasing trend associated with longer duration of use

Table 1.2 Summary of etiologic risk factors, magnitude of effect on endometrial cancer risk, and trends in the prevalence of the risk factor

Risk factor [references]	Magnitude of association	Trend in prevalence of risk factor[a]
Non-contraceptive estrogen-alone use [107]	Estrogen use is associated with a 2.3 times higher EC risk compared with nonuse	↓
Non-contraceptive estrogen plus progestin use [131]	Estrogen plus progestin use is associated with a 22 % lower EC risk compared with nonuse	↓
Tamoxifen use [168]	Tamoxifen use is associated with a 2.7 times higher EC risk compared with nonuse	↓
Sequential oral contraceptive use [171, 173]	Sequential oral contraceptive use is associated with a 4.6–7.3 times higher EC risk compared with nonuse	↓
Combination oral contraceptive use [174]	Combination oral contraceptive use is associated with a 50 % lower EC risk compared with nonuse	Stable
Intrauterine device use [176]	Inert IUD use is associated with a 17 % lower EC risk compared with nonuse	↑
Tubal ligation [185]	No association with EC risk	Stable
Excess adiposity [188]	5 kg/m^2 increase in BMI associated with 1.6 times higher EC risk	↑
Physical activity [204]	Physical activity is associated with a 20–30 % lower EC risk compared with inactivity	↑
Diabetes [211]	Diabetes is associated with a 2.1 times higher EC risk compared with nondiabetics	↑
Metabolic syndrome [220]	Metabolic syndrome is associated with a 1.4 times higher EC risk compared with women without this disease	↑
Early age at menarche [233]	Early age at menarche is associated with a 1.4 times higher EC risk compared with later age at menarche	↑
Late age at natural menopause [233]	Late age at natural menopause is associated with a 2.2 times higher EC risk compared with early age at natural menopause	↑
Parity [154, 260]	Parity is associated with 20–50 % lower EC risk compared with nulliparity	↓
Breastfeeding [133, 233, 240, 251, 260, 264–267]	Insufficient evidence	↑
Infertility [268]	Infertility is associated with a 1.2 times higher EC risk compared with fertile women	↓
Polycystic ovary syndrome [271]	PCOS is associated with a 2.8 times higher EC risk compared with women without this disease	Unknown
Cigarette smoking [272]	Current smoking is associated with a 26–37 % lower EC risk compared with never smoking	Stable
Family history [279]	Family history is associated with a 1.8 times higher EC risk compared with no family history	Unknown

[a]Information available from United States Surveillance programs, including National Health and Nutrition Examination Survey (NHANES), Behavioral Risk Factor Surveillance System (BRFSS), and National Survey of Family Growth (NSFG)

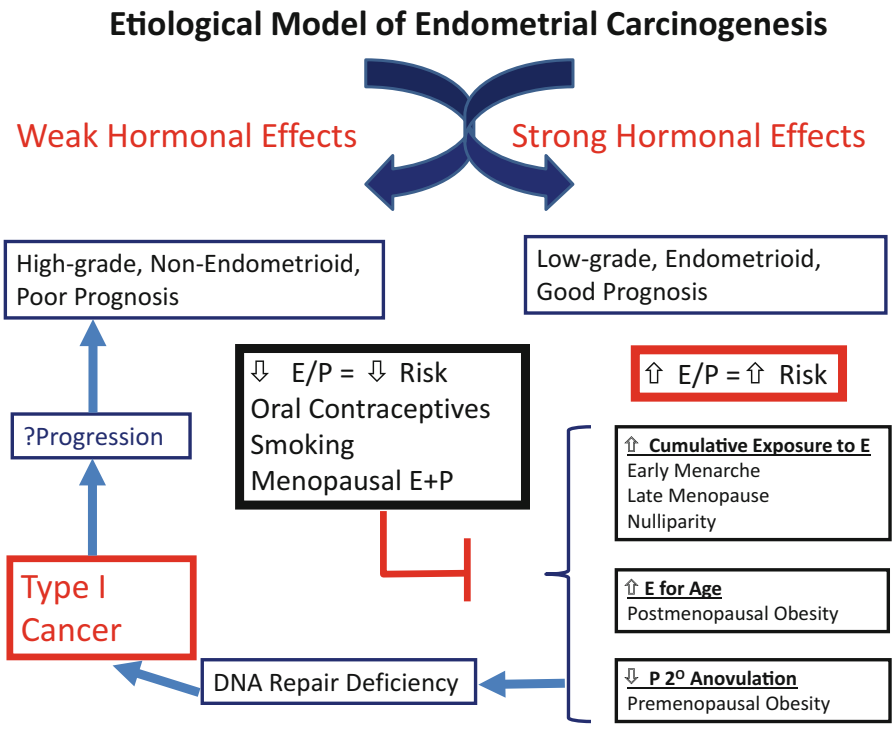

Fig. 1.3 Etiological model of endometrial carcinogenesis

[RR (95% CI), 5–10 years, 5.9 (4.7–7.5); ≥10 years, 9.5 (7.4–12.3)]. Cessation of unopposed estrogen use has been associated with reduction in endometrial carcinoma risk [108–118]; however, only three studies have demonstrated a reduction in risk equivalent to that of nonusers following 2 years of cessation [109, 111, 114]. Other studies indicate that some elevation in endometrial carcinoma risk remains following 3–5 years of cessation of unopposed estrogen [115, 119–121], while some have shown a slightly elevated risk after 10 years of cessation [112, 122–124].

The type of unopposed estrogen therapy has been evaluated in epidemiologic studies with some inconsistency. Conjugated estrogens, the type most commonly prescribed in the United States [125], were linked with higher endometrial carcinoma risk compared with synthetic estrogens in a previous meta-analysis (RR 2.5 vs. 1.3) [107], while other studies have noted similar magnitudes of risk [113, 118, 121, 126–128]. Most studies observed elevated endometrial carcinoma risk at all commonly prescribed doses compared with never use [111, 117, 118, 121, 126, 127, 129, 130]. One study suggested highest endometrial carcinoma risk with the highest dose of conjugated estrogen [121].

Following the recognition that unopposed estrogen use increases endometrial carcinoma risk, progestin (synthetic progesterone) was introduced to counteract endometrial proliferation among women with an intact uterus. Estrogen plus progestin

therapy has varied in the duration that progestin is delivered. Short-duration formulations, also termed sequential or cyclic, provide a progestin component for less than 15 days per month. A meta-analysis reported increased endometrial carcinoma risk associated with progestin prescribed for fewer than 10 days per month [odds ratio (OR) = 1.76 95 % CI = 1.51–2.05], whereas progestin given for more than 10 days per month was unrelated to endometrial carcinoma risk (OR = 1.07, 95 % CI = 0.92–1.24, based on eight studies) [131]. Long-duration formulations, also termed continuous, provide daily progestin and have been linked with lower endometrial carcinoma risk in a meta-analysis of 14 studies (OR = 0.78, 95 % CI = 0.72–0.86) [131].

Effect modification of the postmenopausal hormone use—endometrial carcinoma risk relationship by other endometrial carcinoma risk factors—has been observed. With respect to BMI, the factor most consistently evaluated, some studies have shown that increased risk related to unopposed estrogen use is greatest among normal-weight women, perhaps due to a saturation effect of excess circulating estrogens among obese women [111, 112, 120, 132–139]. Even still, the absolute risk of endometrial carcinoma related to unopposed estrogen is highest among obese women [135, 140]. Similarly, endometrial carcinoma risk is greatest among normal-weight women using sequential estrogen plus progestin [134–136, 139, 141]. Conversely, the greatest risk reduction among users of continuous estrogen plus progestin occurs among obese women [134, 135, 138, 139, 142–145].

Among unopposed estrogen users, increased endometrial carcinoma risk irrespective of smoking status has been observed [108, 112, 140, 146–151]. In one study, smokers who were users of estrogen plus progestin had higher endometrial carcinoma risk than nonsmokers; however, risks were not separately evaluated for sequential vs. continuous regimens [141]. Others have not observed this relationship [134, 135, 142]. Parity has been found to modify risk associated with unopposed estrogen in one study [149] but not others [133, 140, 148, 152–154], while women who used oral contraceptives early in life and unopposed estrogens at older ages had a slightly lower endometrial carcinoma risk in one study [148] but not others [155–158]. Neither parity nor oral contraceptive use has been shown to modify relationships between estrogen plus progestin use and endometrial carcinoma risk [135].

Selective Estrogen Receptor Modulators

The use of the selective estrogen receptor modulator tamoxifen, itself a weak estrogen, has been related to increased endometrial carcinoma risk in two randomized breast cancer chemoprevention trials [159, 160]. Subsequent studies have supported this association [161–166], leading the International Agency on Cancer Research (IARC) to classify tamoxifen as a known human carcinogen [167]. Furthermore, a meta-analysis reported a significantly increased risk of endometrial carcinoma with tamoxifen use (RR = 2.70, 95 % CI = 1.94–3.75) [168]. Tamoxifen has also been linked to increased risk of serous carcinomas and carcinosarcomas in some studies [169, 170], although these tumors are, overall, thought to be less related to sex hormone imbalances.

Contraception Methods

Early contraceptive formulations delivered potent estrogens for 14–16 days per month, followed by a weaker progestin component delivered for 5–10 days per month. Following several reports showing elevations in the RR of endometrial carcinoma ranging between 4.6 and 7.3 [171–173], these preparations were removed from the market.

The use of combined oral contraceptives (COCs), which contain estrogen and progestin taken daily for 21 days per month, is associated with a 50 % lower risk of endometrial carcinoma compared with nonuse [174]. Risk reduction is observed after at least 1 year of use, and increasing duration of COC use is significantly related to progressively greater protection. Furthermore, risk reductions related to COC use have been shown to persist for up to 20 years after discontinuation, suggesting that COCs may be a useful chemopreventive agent providing long-term protection.

Results are mixed regarding the impact of progestin potency on endometrial carcinoma risk. Some suggest that endometrial carcinoma risk is reduced regardless of progestin potency [175], whereas two other studies reported the greatest risk reductions among women using formulations with higher progestin dose [155].

Intrauterine devices (IUDs) have been associated with decreased risk of endometrial carcinoma. In a pooled analysis of four cohort and 14 case–control studies, the use of any type of IUD was related to lower endometrial carcinoma risk (OR = 0.81, 95 % CI = 0.74–0.90) [176], which is in line with two previous meta-analyses [177, 178]. Based on the years of enrollment of studies contributing to the pooled and meta-analyses, risks associated with IUD use likely represent the relationship with inert IUDs. Because the hormone-releasing type of IUD is now the most commonly used IUD in the United States, future epidemiologic studies are needed to investigate a possible association with this type of IUD, which is likely to be more biologically active in the endometrium.

Other contraceptive methods, including injectable contraceptives, implants, and transdermal patches, have been evaluated infrequently in relation to endometrial carcinoma risk [179–182]. As the use of these methods become more prevalent, future studies will be needed to distinguish risks related to exclusive and long-term use of these methods.

Relationships between endometrial carcinoma risk and tubal ligation have been examined in three case–control studies [183–185] and one population-based cohort [186]. Two studies reported a nonsignificantly increased risk of endometrial carcinoma [183, 184], while the other two studies reported moderate, but nonsignificantly, decreased endometrial carcinoma risk [185, 186]. The mechanism is unclear, but potential ovarian devascularization, resulting in reduced total hormone exposure, represents one of several possible explanations.

Excess Adiposity

Obesity is strongly related to endometrial carcinoma risk [187]. In fact, of all obesity-related cancers occurring among women, higher body mass index (BMI) is most strongly related to endometrial carcinoma risk [188]. Epidemiologic studies demonstrate that obese women have a two- to fivefold elevated risk of endometrial carcinoma compared with normal-weight women [189]. These relationships have been observed in both pre- and postmenopausal women as well as in cohort and case–control studies.

Studies that model BMI continuously report a linear relationship between BMI and endometrial carcinoma risk. For example, in a meta-analysis of 19 cohort studies, Renehan et al. [188] reported the overall RR of endometrial carcinoma to be 1.59 times higher for each 5 kg/m^2 increase in BMI. In the Million Women Study conducted in the United Kingdom, investigators found that increasing BMI was associated with increased incidence of endometrial carcinoma (trend in RR per 10 units, 2.89; 95 % CI, 2.62–3.18) [190]. Additionally, a recent retrospective cohort study of overweight and obese women undergoing hysterectomy demonstrated a linear relationship between increasing BMI and endometrial carcinoma risk: each 1 kg/m^2 increase in BMI was associated with an 11 % increase in the proportion of patients diagnosed with endometrial carcinoma [191]. Further, each 5 kg increase in adult weight gain was associated with a 39 % increase in postmenopausal endometrial carcinoma risk among nonusers of menopausal hormones (95 % CI = 1.29–1.49) [192]. Among menopausal hormone users, the linear association was observed albeit attenuated (RR = 1.09, 95 % CI = 1.02–1.16). This finding is unsurprising in light of data suggesting that endometrial cells experience their highest mitotic activity when estradiol levels are approximately 50 pg/ml—further increases in estradiol may not result in greater endometrial cell proliferation [193].

Other anthropometric measures, including waist circumference, hip circumference, waist/hip ratio, and waist/height ratio, have been suggested as endometrial carcinoma risk factors [143, 144, 194–200]. Unlike BMI, which is an indicator of total body weight, these measures are thought to better reflect central adiposity. Different adipose compartments may vary in their effects on hormone levels and other factors. Most studies report positive associations between endometrial carcinoma risk with the various body fat distribution measures, which is subsequently attenuated after adjusting for BMI [143, 144, 194, 196, 198].

Evidence for associations between obesity and endometrial carcinoma risk among subgroups of other endometrial carcinoma risk factors was recently synthesized [187]. The categories of overweight and obese were collapsed into an excess body weight category. Although excess body weight was associated with increased endometrial carcinoma risk in most subgroups, some notable differences were observed. Excess body weight was a stronger predictor of risk among nonsmokers (RR = 2.69, 95 % CI = 1.35–2.13) compared with smokers (RR = 1.57, 95 % CI = 1.27–1.93) as well as among diabetics (RR = 2.09, 95 % CI = 1.72–2.54) compared with nondiabetics (RR = 1.50, 95 % CI = 1.25–1.79). Notably, effect estimates

comparing hormone users and nonusers were similar (RR = 1.48 vs. 1.69); however, the type of hormone formulation (pure estrogen versus estrogen plus progestin) was not considered which likely led to similar effect sizes.

Postmenopausal obesity is associated with increased circulating estrogens, attributable to aromatization of androgens in adipose tissue [30, 201]. Obesity is related to lower levels of SHBG, leading to higher bioavailable levels of estrogen and higher insulin levels, which may elevate endometrial carcinoma risk [35, 202]. Other nonhormonal mechanisms for the obesity–endometrial carcinoma association include inflammation and other metabolic pathways (*reviewed in later section*). Among premenopausal women, where estrogen levels are high regardless of BMI, obesity may lead to a greater frequency of anovulatory cycles and relative progesterone deficiency or increased inflammation, which could contribute to increase risk of developing endometrial carcinoma.

Physical Activity

Four meta-analyses [203–206], which summarized 14 cohort and 12 case–control studies, have reported that moderate physical activity is associated with a 20–30 % reduction in endometrial carcinoma risk, regardless of domain (occupational, recreational, household, transport). Adjustment for BMI or other indices of weight attenuates but does not abolish this relationship. One meta-analysis [206] addressed potential dose–response relationships between increasing physical activity and endometrial carcinoma risk and reported that an increase in three metabolic equivalent of task (MET) hours/week was associated with a 2 % decreased risk of endometrial carcinoma (RR = 0.98, 95 % CI = 0.95–1.00, $p = 0.02$), while an increase of 1 h/week in physical activity was related to a 5 % lower risk of endometrial carcinoma (RR = 0.95, 95 % CI = 0.93–0.98, $p < 0.001$). Independent of physical activity, sedentary time has been linked with increased endometrial carcinoma risk in a meta-analysis [207]. Endometrial carcinoma risk was significantly higher in women with the highest vs. lowest levels of sedentary behavior (RR = 1.36, 95 % CI = 1.15–1.60).

Physical activity is likely to mediate endometrial carcinoma risk, in part, by enabling weight control and reducing adipose stores, the major site of postmenopausal estrogen synthesis. Further, physical activity is associated with higher SHBG levels, leading to less bioavailable estrogen. Importantly, physical activity in the absence of weight loss has been linked with lower levels of estrogen and improved insulin sensitivity, although the effects are larger with greater loss of body fat [208, 209]. Given that physical activity has been linked with lower endometrial carcinoma risk independent of BMI [206], other biological pathways, including inflammation, immune function, and cell signaling pathways [205], might be affected by physical activity.

Diabetes

Three meta-analyses have demonstrated increased endometrial carcinoma risk associated with diabetes [210–212]. Importantly, a question of BMI independence remains, given that some studies did not adjust for BMI, which is related to increased risk of both endometrial carcinoma and diabetes. Of the studies included in the syntheses, two cohort studies [213, 214] and one case–control study [215] observed BMI-independent effects of diabetes on endometrial carcinoma risk, which ranged from 1.43 to 1.94. Furthermore, some studies suggest that risk associated with diabetes is strongest in the category of overweight or obese women compared with normal-weight women [116, 213, 215, 216]. For example, one study reported that the RR associated with diabetes among non-obese women was 1.75 (95 % CI = 0.93–3.30), whereas in obese women, the RR was 6.39 (95 % CI = 3.38–12.06), although the interaction of diabetes and BMI was not significant [213]. Two case–control studies [217, 218] and one cohort study [219] have evaluated risk of endometrial carcinoma in relation to metformin, an antidiabetic medication, all of which were null.

Diabetes has been hypothesized to affect endometrial carcinoma risk through several mechanisms that increase endometrial proliferation, including increasing mediators of endometrial proliferation [estrogen and insulin-like growth factors (IGFs)], or by decreasing levels of the corresponding binding proteins (SHBG and IGFBP), which increases the bioavailability of these factors (*reviewed in later section*).

Metabolic Syndrome

Metabolic syndrome, which represents a constellation of factors, including obesity, hypertension, insulin resistance, and dyslipidemia, has been linked with increased endometrial carcinoma risk [216, 220–225]. In the largest study to evaluate this relationship (16,323 endometrial carcinoma cases and 100,751 controls), a 40 % increased risk of endometrial carcinoma was observed (OR = 1.39, 95 % CI = 1.32–1.47) [220]. Given the strong relationships between high BMI and endometrial carcinoma risk, efforts to evaluate the relative importance of the other metabolic syndrome components suggest that while BMI is the strongest risk predictor, hypertension and high triglycerides retain statistical significance in mutually adjusted models, albeit with smaller magnitudes of effect.

Metabolic syndrome is likely to increase endometrial carcinoma risk by affecting multiple biologic pathways, including estrogen and progesterone levels, inflammatory cytokines, and insulin (*reviewed in other sections*).

Ages at Menarche and Menopause

Younger age at menarche has been linked with increased endometrial carcinoma risk in some [132–134, 147, 226–237] but not all studies [113, 238–242], whereas older age at menopause has consistently been associated with increased endometrial carcinoma risk [132, 133, 147, 226–233, 236, 237, 239, 241]. A potentially more biologically relevant construct is menstruation span or the interval between menarche and menopause. In a population-based case–control study, a dose–response relationship between endometrial carcinoma risk and increasing years of menstruation was observed: compared with less than 30 years of menstruation, 40 or more years of menstruation were associated with an OR of 2.71 (95 % CI = 1.67–4.40, *p*-trend <0.01) [243]. This association may reflect risk related to exposing the endometrium to a greater cumulative number of proliferative cycles, which in turn increases risk of acquiring mutations.

Parity and Related Factors

Parity and gravidity, which refer to the number of live births and pregnancies, respectively, are associated with decreased endometrial carcinoma risk. Most studies report a 20–50 % risk reduction for parous vs. nulliparous women [116, 132–134, 147, 148, 154, 171, 226, 227, 229, 231–233, 236, 238–240, 243–260], with further reductions in risk associated with an increasing number of live births among parous women [116, 132–134, 147, 148, 171, 226, 227, 232, 233, 238–240, 244, 247, 249, 253–258]. An analysis that evaluated associations between endometrial carcinoma and hormone-related risk factors by parity status did not identify differences between nulliparous vs. parous women [154].

Relationships between timing of births and endometrial carcinoma risk are less consistent. Some studies have shown older age at first birth is related to lower endometrial carcinoma risk [230, 251, 256, 258], higher endometrial carcinoma risk [240], or no association [133, 171, 231, 239, 243, 244, 249, 250, 255, 260–262]. In a pooled analysis including 8,671 endometrial carcinoma cases and 16,562 controls, the combined OR per 5-year increase in age at last birth was 0.88 (95 % CI = 0.85–0.91) [263].

Associations between induced or spontaneous abortions and endometrial carcinoma risk are mixed: induced abortion has been linked with increased risk [231, 260], lower risk [226, 249, 256], or no association [133, 229, 233, 251], whereas spontaneous abortions have not been associated with risk in some [133, 226, 227, 229, 231, 240] but reduced risk in one [249].

Effects of breastfeeding, which may further suppress estrogen exposure, on endometrial carcinoma risk are inconclusive. Studies conducted in Western countries, where cumulative breastfeeding duration is relatively low, have been null [133, 233, 260, 264]. Conversely, studies conducted in countries where breastfeeding duration is typically longer have reported decreased endometrial carcinoma risk associated with longer breastfeeding duration [240, 251, 265–267].

Infertility has been linked with endometrial carcinoma risk in a recent pooled analysis including 8153 endometrial carcinoma cases and 11,713 controls [268]. Infertile women (assessed mainly by self-report) had an increased risk compared with those without infertility concerns, even after accounting for nulliparity (OR = 1.22; 95 % CI = 1.13–1.33).

Pregnancy is associated with higher levels of progesterone-relative estrogen, which may account for its protective effect. In addition, endometrial shedding during birth may offer protection via exfoliation of premalignant or initiated cells. The suggestion that older age at last birth, which should be associated with more recent births, is protective has been presented in support of the exfoliation theory [244, 249].

Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is characterized by a constellation of abnormalities that increase risk of endometrial carcinoma, including, chronic anovulation, obesity, and diabetes [250]. Prolonged anovulation is accompanied by progesterone deficiency, which is thought to be a key factor in endometrial carcinogenesis among premenopausal women [269]. Although an association between PCOS and cancer has been discussed since the 1940s [270], epidemiological evidence supporting the link is limited. A meta-analysis of data from five epidemiological studies reported that women with PCOS were at a significantly increased risk of endometrial carcinoma (OR = 2.79, 95 % =1.31–5.95) [271]. Importantly, various definitions of PCOS are used throughout the literature, which complicate interpretation. Further, efforts to disentangle the effects of PCOS from its component factors, obesity and insulin resistance, are difficult.

Cigarette Smoking

A consistent inverse relationship between cigarette smoking and endometrial carcinoma risk has been observed in the literature; one meta-analysis demonstrated that current smokers have a 26 % (95 % CI = 0.64–0.84) lower risk in cohort studies and a 37 % lower risk in case–control studies (95 % CI = 0.55–0.72) [272]. The inverse association was demonstrated among postmenopausal, but not premenopausal women. A relationship between more cigarettes per day and lower endometrial carcinoma risk confirms a dose–response relationship; however, relationships between longer duration and younger ages at initiation were not statistically significant in prospective studies [272]. The mechanism by which cigarette smoking reduces endometrial carcinoma risk is unknown; however, some hypothesized antiestrogenic mechanisms, including increased production of 2-hydroxyestrone, which is postulated to be anticarcinogenic [273, 274] and higher progesterone levels in endometrial tissues and in the circulation

[275, 276]. Smokers and nonsmokers do not differ with respect to serum estrogen levels [277]; however, urinary excretion of estriol is lower in smokers than in nonsmokers [278].

Family History

First-degree family history of endometrial carcinoma is associated with a higher risk of developing endometrial carcinoma compared with individuals lacking a family history. A recent meta-analysis, which included 2339 endometrial carcinoma cases and 16,000 controls, reported an 82 % higher risk (95 % CI = 1.65–1.98) [279]. Cumulative risk of endometrial carcinoma, up to age 70 years, was estimated at 3.1 % (95 % CI 2.8–3.4) for women with a first-degree relative with endometrial carcinoma with a population-attributable risk of 3.5 % (95 % CI 2.8–4.2). This analysis did not find evidence of effect modification by age at diagnosis, by menopausal status, or by the affected family member (i.e., sister vs. mother), although individual studies have reported stronger effects among younger women [171, 256, 280].

Family history of cancer can reflect shared environments or inherited genetic conditions. Inherited predisposition to endometrial carcinoma has been estimated at 5 % [280], with Lynch syndrome accounting for the majority of inherited endometrial carcinomas [281]. Lynch syndrome is characterized by deleterious germline mutations in the DNA mismatch repair genes, *MSH2, MSH6, MLH1,* and *PMS2*, which result in faulty mismatch repair of errors that occur during DNA replication, manifested as microsatellite instability, detection of abnormal lengths of short repetitive DNA sequences [282]. Women with germline mutations in either *MLH1* or *MSH2* have a 40–60 % lifetime risk of developing endometrial carcinoma [283, 284]. Recently, it has also been discovered that specific germline variants in the *POLD1* gene, which encodes a DNA polymerase, also predispose carriers to develop endometrial cancer in the context of polymerase proofreading-associated polyposis [285, 286].

Genetic Risk of Endometrial Carcinoma

Candidate gene studies (reviewed [287]) have reported on the association between common single nucleotide polymorphisms in several biological pathways, such as sex steroid hormone [288–295] and obesity [296–298], in relation to endometrial carcinoma risk, although not all studies found significant associations. In addition, agnostic evaluations of the relationship between common genetic variants and endometrial carcinoma risk have been conducted using the genome-wide association study (GWAS) approach [299–301]. These efforts have identified a novel candidate locus, rs4430796, at the *HNF1B* gene region on chromosome 17q12 [299], but subsequent studies did not establish a link with endometrial carcinoma risk that reached genome-wide significance [301, 302]. Further, an exome-wide association study did not find rare variants associated with endometrial carcinoma risk [303].

Other Risk Factors

Studies evaluating diet, alcohol, nonsteroidal anti-inflammatory drugs, endometriosis, uterine fibroids, pelvic inflammatory disease, and sexually transmitted infections as possible endometrial carcinoma risk factors have yielded uncertain conclusions [304–308]. Meta-analyses of the existing data are appropriate for certain risk factors, whereas additional studies are needed for sparsely investigated risk factors.

Etiologic Heterogeneity

The risk factor relationships described in this section are most applicable to the prevalent type I tumors. A number of studies have investigated relationships between the established endometrial cancer risk factors and incidence of histologic subtypes [18, 309–312]. Taken together, these studies demonstrate that factors related to endometrial cancer risk overall are also associated with risk of the individual histologic subtypes. However, the magnitude of associations differs. For example, relative to controls, obesity (BMI $\geq$ 40 kg/m^2) was associated with higher risk of endometrioid (RR = 6.88, 95 % CI = 5.95–7.96), serous (RR = 2.85, 95 % CI = 1.80–4.52), clear cell (RR = 4.36, 95 % CI = 2.16–8.82), mucinous (RR = 3.29, 95 % CI = 1.51–7.19), and mixed tumors (RR = 3.49, 95 % CI = 2.06–5.90) [312]. The overlap in risk factor associations between histologic subtypes supports the need for molecular classification of endometrial carcinomas to develop improved risk factor profiles for specific tumor subtypes.

Non-estrogenic Mechanisms of Endometrial Carcinogenesis

Elevated endogenous estrogens may not fully account for the endometrial carcinoma association with obesity, the strongest risk factor for endometrial carcinoma. Mounting evidence from epidemiologic studies suggests that metabolic and endocrinologic abnormalities, reflected in elevated androgens, insulin, inflammatory mediators, and adipokines, may also contribute to endometrial carcinoma risk among obese women. Several of these factors, such as insulin resistance, increased levels of leptin, decreased levels of adiponectin, and chronic inflammation, are proposed to be important in obesity-related carcinogenesis (mechanisms reviewed in [29, 313]).

Androgens are hypothesized to play a role in endometrial carcinogenesis through their conversion to estrogen by aromatase in the adipose tissue after menopause [32]. However, it is currently not clear whether androgens also have a direct effect on the etiology of endometrial carcinoma [314–316]. Data from a case–control study (n = 276 endometrial carcinoma cases) showed that higher serum levels of androstenedione were associated with a two- to threefold elevated risk of endometrial carcinoma in pre- and postmenopausal women, even after adjusting for levels

of estrogen [317]. In contrast, more recent nested case–control studies ($n = 124$ and 247 endometrial carcinoma cases) reported that elevated levels of androstenedione were not associated with risk [34] or this risk disappeared after adjusting for estrogen [33]. Increased endometrial carcinoma risk was also observed with elevated testosterone levels [33, 34] and with DHEAS in one study [33] but not another [34].

A pronounced metabolic change associated with obesity is the development of insulin resistance, which is linked with higher levels of circulating insulin (also referred to as hyperinsulinemia) [29, 313]. Insulin is a known mitogen, and endometrial tissues express high-affinity insulin receptors, which are consistent with a direct effect of insulin on endometrial cancer cells in culture [318, 319]. Further, cell line studies have shown that insulin, through its regulation of IGFBP1, increases IGF1 activity in the endometrium [320, 321]. Insulin and IGF share extensive amino acid sequence homology and use a common PI3K (phosphoinositide kinase-3)/AKT/mTOR signaling pathway that promotes cell survival and proliferation [322]. Insulin is described to also suppress levels of SHBG, leading to higher levels of bioactive estrogen.

Epidemiologic evidence has consistently supported a positive relationship between overall endometrial carcinoma risk with higher levels of insulin [35, 323–325] and C-peptide (a stable marker of pancreatic insulin secretion) [202, 326, 327]. Fewer studies have reported on free IGF1 levels, with some reporting an inverse association, albeit an inconsistently statistically significant relationship [35, 324, 326, 328–331]. However, epidemiological studies reporting on the possible association with serum levels of different isoforms of IGFBP have been inconclusive [35, 324, 325, 328–332].

Inflammation has also been implicated in endometrial carcinoma etiology. Chronic inflammation can induce cell division, increasing the possibility of replication error and ineffective DNA repair, and directly increase estrogen production [333]. Few epidemiological studies have investigated the association between risk of endometrial carcinoma and inflammatory markers, namely, IL-1 receptor antagonist (IL-1RA) [334], C-reactive protein (CRP; [323, 334, 335]), interleukin (IL)-6 [323, 334, 335], and tumor necrosis factor (TNF)-α [323, 335, 336]. Among these inflammatory markers, an increased level of CRP has been most consistently associated with elevated risk of endometrial carcinoma [323, 334, 335]. The risk association was statistically significant, even after adjusting for BMI alone or adjusting for BMI, estradiol [335], and markers of insulin separately [323, 335], albeit the association was slightly attenuated after the adjustments. These data indicate that inflammation, in addition to elevated estrogen and hyperinsulinemia, may provide the link between obesity and endometrial carcinoma risk.

Adipose tissue is considered an endocrine organ that secretes a large range of proteins. Of interest, an altered level of cytokines, known as adipokines, such as adiponectin and leptin, has been associated with adipose tissue dysfunction [313]. Previous case–control studies have reported that low adiponectin level is associated with endometrial carcinoma, even after controlling for BMI [337–340]. Fewer numbers of case–control studies nested within prospective cohort studies have been evaluated and have reported inconsistent results: two studies reported an inverse association [332, 341],

whereas the other two reported no association [342, 343]. Results from case–controls studies that suggested a positive association between increased leptin levels [337, 338, 344] and elevated endometrial carcinoma risk have been confirmed in two prospective studies using pre-diagnostic levels of adiponectin in serum [341, 342]. Three prospective studies evaluating the leptin to adiponectin ratio observed that a higher ratio was associated with elevated risk of endometrial carcinoma [340–342]. One study that evaluated the association between visfatin in relation to endometrial carcinoma risk did not find an association [341]. Recently, a factor analysis of various pre-diagnostic plasma hormones, binding proteins, and cytokines in 233 endometrial carcinoma cases and 446 matched controls identified three relatively independent and physiologically well-defined pathways that were associated with postmenopausal endometrial carcinoma risk: steroids, insulin resistance/metabolic syndrome, and inflammation [336]. Serum profiling of a panel of metabolic dysfunction analytes in a case–control analysis (15 amino acids and 45 acylcarnites) has also identified candidate serum biomarkers associated with endometrial cancer, but confirmation in prospective data has not been published to date [345].

Summary and Future Directions

The total number of endometrial carcinoma cases in high-income nations is increasing secondary to growing populations, extended life expectancy, reduced performance of hysterectomy, and increasing obesity. The slow development of most endometrial carcinomas from recognized precursors suggests the potential for early detection or preventive interventions to improve clinical outcomes. However, better methods to identify women at greatest risk of developing endometrial carcinoma would enable more efficient testing of new approaches. Toward that goal, efforts to develop useful models to predict risk of endometrial carcinoma are needed [346]. Given that obesity is a strong risk factor for endometrial carcinoma, but also extremely prevalent, understanding which obese women are at greatest risk may contribute importantly to the success of this effort. Improved etiological understanding of endometrial cancer has enabled the development of targeted prevention trials that include interventions such as levonorgestrel-impregnated intrauterine devices, metformin, and weight loss [347].

Finally, efforts to detect endometrial carcinoma at early stages (and potentially at the precursor stage) using molecular testing of cervical cytology samples or tampons [348–350] have shown preliminary promise and may help bridge identification of high-risk populations, enabling timely interventions and reduction in mortality. Given the expected increases in endometrial cancer incidence, streamlined clinical triage will be important; abnormal vaginal bleeding is among the most frequent gynecologic complaints, and although benign in the vast majority of cases, identifying the subset of women who have early carcinomas or precursors could reduce mortality and lessen treatment-related morbidity.

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Chapter 2
Clinical Behavior and Treatment of Endometrial Cancer

Divya Gupta

Abstract Endometrial cancer is the most common gynecologic malignancy diagnosed in women in the developed nations. It affects a disproportionate number of reproductive-aged women. While the overall prognosis is good compared to other cancers affecting women, the pathogenesis and clinical behavior of endometrial cancer are heterogeneous. The risk factors associated with the type I and type II endometrial cancers and their pathogenesis will be discussed, as well as the evaluation and primary treatment of women with endometrial cancer. The chapter will also focus on risk stratification for recurrence after surgery and role of adjuvant treatments. Finally, the treatment of recurrent endometrial cancer will be presented.

Keywords Endometrial cancer • Risk factors • Lynch syndrome • Fertility sparing • Chemotherapy • Radiation • Surgery • Minimally invasive surgery • Lymphadenectomy

Epidemiology

Endometrial cancer (EC) is the most common gynecologic cancer found in women in the developed nations. An estimated 54,870 new cases of uterine cancer will be diagnosed, and approximately 10,170 deaths due to EC will occur in the United States in 2015 [1]. Throughout the world, there are an estimated 319,500 incident uterine cancers reportedly annually, which account for over 76,000 deaths each year [2]. In the United States, the incidence of EC is increasing among Black women, Asian Pacific islanders, and Hispanics [1]. Although Black women experience a lower incidence of endometrial carcinoma, they are more than twice as likely to die from the disease as White women [1]. Black women are often diagnosed with high-grade or type II endometrial carcinomas, to be discussed later in this chapter. For a more thorough discussion of the epidemiology and risk factors of endometrial cancer, see Chap. 1.

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Risk Factors

The risk factors can be divided among three different categories: reproductive factors, hormonal use, and others. Reproductive risk factors include nulliparity, early menarche, late menopause, infertility, and anovulatory menstrual cycles [3]. States of excess estrogen and progesterone are associated with hormone-responsive endometrial cancers. These include unopposed estrogen use such as in estrogen-only use in women with an intact uterus, selective-estrogen receptor modulator (SERM, such as tamoxifen, raloxifene) use, polycystic ovarian syndrome (PCOS), and obesity. Population-controlled studies indicate lack of physical activity and comorbid conditions, such as diabetes and metabolic syndromes, also increase the risk of endometrial cancer development. Protective risk factors include breastfeeding, use of combined oral contraceptive, levonorgestrel intrauterine device, and cigarette smoking. Those risk factors are often related as obesity, infertility, anovulatory menstrual cycles, and polycystic ovarian syndrome may co-exist [3]. Some of the reproductive risk factors for endometrial cancer are similar to those for breast cancer, and breast cancer patients taking SERMs have a slightly higher risk of endometrial cancer. In contrast, women with high-risk pathologic subtypes of EC don't exhibit these classic risk factors. They tend to be older and thinner and may have a family history indicating increased risk.

There are several hereditary syndromes associated with EC. The most common is Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer [4, 5]. The syndrome is characterized by inheritance of germ line mutations in the following DNA mismatch repair (MMR) genes: *MSH2, MLH1, MSH6,* and *PMS2*. Individuals with Lynch syndrome have a germ line mutation in one of the MMR genes. During their lifetime, the second allele may be inactivated via mutation, loss of heterozygosity, or epigenetic silencing by promoter hypermethylation. This results in lack of functional DNA repair leading to mutations, usually at particular repeated nucleotide sites called microsatellites (see Chap. 4). Accumulation of the DNA errors leads to the development of cancer. In the order of frequency, women with Lynch syndrome have a high risk of developing colorectal, endometrial, ovarian, urologic, gastric, small bowel, pancreatic, and brain tumors. Up to 70 % of women will develop endometrial cancer, which is usually hormonally responsive, and, in half of the cases, EC is the incident cancer in a Lynch syndrome family [4, 5]. Cowden syndrome is a second autosomal dominant syndrome associated with germ line *PTEN* mutations. *PTEN* is a tumor suppressor which negatively regulates the phosphatidylinositol 3-kinase AKT and mammalian target of rapamycin (mTOR)-signaling pathways, which are critical for cell proliferation, cell cycle progression, and apoptosis and are commonly found to be mutated in EC. Women with Cowden syndrome develop mucocutaneous lesions, breast cancer, EC, medullary thyroid cancer, and genitourinary malignancies [6]. Mutations in *BRCA 1–2* have shown an inconsistent association with uterine cancer, specifically uterine serous carcinoma [7, 8].

While the peak incidence of endometrial cancer is in postmenopausal women, ages 60–70, approximately 15 % of cancers are identified in women less than 45 years of age [9]. For this subset of women, fertility concerns are paramount

along with treatment of a malignant condition. Most of these women have estrogen- and progesterone-responsive endometrial cancers, and fertility-sparing treatments are a consideration.

Diagnosis of Endometrial Cancer

Most women with endometrial cancer initially present with abnormal uterine bleeding. They are diagnosed by an endometrial biopsy performed in the office or an operative dilation and curettage (D&C). These procedures result in a concordant histopathologic diagnosis in 99 % of cases [10]. All women with postmenopausal bleeding should be initially evaluated with an endometrial biopsy regardless of risk factors or hormone use. In premenopausal or perimenopausal women, an endometrial biopsy is recommended based on risk factors for malignancy: obesity, PCOS, evidence of anovulation or unopposed estrogen, persistent abnormal bleeding, or family history [11]. The role of a hysteroscopy, distention of the endometrial cavity with a sterile solution and visualization with an endoscopic camera, at the time of an operative D&C is controversial. Theoretically, there is a concern that any malignant cells in the endometrial cavity can spread into the peritoneum via the fallopian tubes. Small retrospective studies have presented mixed data on the prevalence of malignant peritoneal cytology in women who had previously undergone hysteroscopy. Clinically, malignant peritoneal cytology has little effect on overall prognosis or survival in endometrial cancer. Currently, there are no guidelines to recommend or discourage hysteroscopy during an operative D&C [12].

A transvaginal ultrasound is another diagnostic tool used in the evaluation of women with postmenopausal bleeding or abnormal menstrual bleeding. In postmenopausal women, an endometrial thickness of greater than 4 mm has an 85 % positive predictive value with 96 % specificity and 100 % sensitivity for endometrial abnormalities [13]. This can be a useful diagnostic tool to evaluate a patient prior to performing a biopsy or to determine if an operative procedure is needed if an office procedure is unsuccessful. Regardless of pelvic ultrasound findings, if a woman has persistent postmenopausal bleeding or abnormal menstrual bleeding, a histologic diagnosis is required.

Exams such as cervical cytology or routine pelvic ultrasounds are not recommended for EC screening due to high false-positive and false-negative rates and prohibitive costs. The diagnosis of atypical glandular cells NOS or favor neoplasia using the Bethesda classification on a cervical cytology is associated with a 7 % risk of endometrial hyperplasia or cancer [14]. Women showing this cytologic result are evaluated for both endometrial and cervical pathology with biopsies. Women at high risk of developing endometrial cancer, such as those with Lynch syndrome or Cowden disease, are recommended to undergo a screening ultrasound starting at age 30 in addition to endometrial biopsy [15, 16]. In pre- and postmenopausal women, evidence of intrauterine polyps is an indication for an ultrasound and possible D&C/hysteroscopy. In postmenopausal women with asymptomatic thickening of the endometrial lining or polyps, i.e., no irregular bleeding, the risk of occult endometrial pathology is 3–5 % [17].

Pretreatment Evaluation

Once endometrial cancer has been diagnosed, pretreatment evaluation includes a full history and physical examination, discussion of hereditary risk factors and family history, pretreatment imaging, and medical evaluation.

Discussion of medical and family history is important to develop a presurgical plan and consider genetic testing for those with strong risk factors. Given the risk factors of obesity, diabetes, metabolic syndrome, and older age, many women have comorbidities that can determine treatment planning. A careful preoperative evaluation, especially cardiopulmonary evaluation and good glycemic control, is important to achieve good perioperative outcomes. Management of obesity and related diseases, such as pulmonary hypertension and chronic obstructive sleep, should be optimized. Patients with family histories concerning for Lynch syndrome or Cowden disease are also recommended to seek genetic counseling for possible germ line testing. While this may not change the recommendation for primary treatment, it is important in subsequent therapy, overall prognosis, prevention of other cancers, and discussion with at-risk family members. In addition, if an incidental colorectal cancer is diagnosed on a preoperative colonoscopy, it can be surgically excised at the time of hysterectomy. Lynch testing is recommended for women diagnosed with EC < age 50 whose tumor biopsy shows loss of MMR gene expression by immunohistochemistry.

Presurgical imaging is recommended for treatment planning. A pelvic ultrasound is usually sufficient imaging for someone with grade 1 endometrial cancer given the low risk of extrauterine spread. In patients with grade 2 or 3 disease or systemic symptoms, such as abdominal distention and palpable masses on abdominal or pelvic exams, computed tomography (CT) imaging with intravenous contrast dye is recommended to evaluate for extrauterine disease [18]. Chest imaging with plain radiography or CT are recommended for those with high-risk pathologies or evidence of cardiopulmonary symptoms. In those patients with grade 3 or other high-risk pathologies, preoperative evaluation of pelvic and periaortic lymph nodes is an important aspect of pretreatment evaluation. Grossly enlarged lymph nodes could determine the mode of surgical treatment along with surgical cytoreduction. Magnetic resonance imaging and FDG-PET/CT are more sensitive than CT for determining positive lymphadenopathy on imaging [19, 20]. A multi-institutional prospective trial also evaluated the role of PET/CT followed by lymphadenectomy to determine the sensitivity and specificity of this imaging modality. Results are still pending maturity. At the current time, data is limited for the use of PET/CT imaging in the primary evaluation of endometrial cancer unless there are patient factors that limit the use of intravenous contrast dye, such as renal disease or hypersensitivity reactions.

Pathology and Disease Stage

Overall prognosis of patients with endometrial cancer depends on two main factors: disease stage and histopathology. Endometrial cancer is surgically staged as per the 2009 International Federation of Gynecology and Obstetrics (FIGO) staging

Table 2.1 2009 International Federation of Gynecology and Obstetrics (FIGO) staging criteria for uterine carcinoma

Stage	Substage	Definition
I		Tumor confined to the uterus
	IA	Tumor confined to endometrium or invades <50 % myometrium
	IB	Tumor invades ≥50 % myometrium
II		Tumor invades stromal connective tissue of the cervix but does not extend beyond uterus
III		Tumor spread to adnexa, serosa, peritoneal lymph nodes
	IIIA	Tumor involves serosa and/or adnexa
	IIIB	Tumor involves vaginal or parametrium
	IIIC1	Tumor spread to pelvic lymph nodes only
	IIIC2	Tumor spread to periaortic lymph nodes
IV		
	IVA	Tumor invades bladder or rectal mucosa
	IVB	Distant metastases, including upper abdomen and lymphatics outside the peritoneum

Table adapted from the FIGO guidelines [21]

criteria [21] (Table 2.1). This involves a systematic procedure, involving a total hysterectomy (TH), bilateral salpingo-oophorectomy (BSO), pelvic and para-aortic lymphadenectomy, and collection of peritoneal cytology. The final surgical stage is categorized from I to IV: I, uterus-confined tumor; II, involvement of the cervix; III, adnexal or lymph node involvement; and IV, all other metastatic sites. Tumor spread can be via local organ involvement, lymphatic, and hematogenous. Before 2010, FIGO staging divided stage I disease into IA (no myometrial invasion), IB (<50 % myometrial invasion), and IC (≥50 % myometrial invasion).

The FIGO staging was, in part, developed due to the importance of the histopathological types of endometrial cancer. The different subtypes of endometrial adenocarcinoma include the following: endometrioid, serous, clear cell, carcinosarcoma or malignant mixed mullerian tumor (MMMT). Diagnostic dilemmas, such as mixed tumors and complex atypical hyperplasia, will also be discussed in the subsequent sections.

Type I vs. Type II Endometrial Adenocarcinoma

Endometrial cancer has been broadly categorized into type I and type II over the past three decades based on an initial clinicopathologic study followed by molecular analyses [22]. Sherman et al. have described the molecular basis of this categorization in previous chapters. Although next generation sequencing studies (see Chap. 5) are beginning to refine this model, much of the information discussed in this chapter has been acquired based on this classification system. Briefly, type I tumors are endometrioid type, which arise in states of excess estrogen.

These include grades 1–3 endometrioid endometrial adenocarcinomas (EEC) and are typically diagnosed at early stages with long-term survival rates >90 %. These tumors often express estrogen and progesterone receptors. At a molecular level, they have a high frequency of mutations in *PTEN, PIK3CA, ARIDIA*, *KRAS, AKT, and mTOR* genes as seen in the TCGA analysis [23]. EEC tumors have a well-defined precursor lesion, complex atypical hyperplasia (CAH), which arises in states of unopposed estrogen such as obesity, estrogen-only use, and polycystic ovarian syndrome (PCOS). While the majority of type I tumors present at an early stage (stages I–II), some are advanced due to local, lymphatic, or hematogenous spread. In these cases, treatment recommendations are similar to type II carcinomas and will be discussed in the next section.

In comparison, type II tumors include uterine serous carcinoma (USC), uterine clear cell (UCC), and MMMT that have high-grade morphological features [22, 23]. These account for 10–20 % of all endometrial adenocarcinomas diagnosed worldwide. They are often characterized by *TP53* and *PIK3CA* mutations and an abnormal DNA content. Altogether, type II endometrial cancers account for the majority of treatment failures, metastatic disease, and deaths related to endometrial cancer.

Uterine serous carcinoma (USC), the most common of the type II endometrial cancers, was described as a distinct entity from EEC in 1982 [24]. USC is histologically similar to serous epithelial tubal/ovarian carcinoma with a propensity for peritoneal spread and approximately 40 % chance of being diagnosed with stage III or IV disease. Stage for stage, USC is associated with a worse prognosis than EEC [25]. While representing less than 10 % of all endometrial cancer cases, USC accounts for 40 % of all endometrial cancer-related deaths [25]. In USC, in contrast to EEC, the risk of extrauterine spread remains high despite the absence of traditional risk factors such as deep myometrial invasion (MI) or lymphovascular space invasion [26, 27]. A precursor lesion to USC, serous endometrial intraepithelial carcinoma (SEIC) has also been identified. While SEIC is considered a preinvasive lesion, it has been associated with a 40 % risk of extrauterine disease in the absence of myometrial invasion and high risk of peritoneal recurrence [26, 27].

Clear cell tumors of the uterus (UCC) are perhaps the least understood pathologic subtype. While clear cell tumors are also found in the ovary and the renal system, they are rare, and molecular analysis of each subtype has demonstrated that they are likely different tumors overall. Like other type II tumors, clear cell tumors have a propensity to be diagnosed at late stages with extrauterine spread. Unlike USC, they tend to be more resistant to adjuvant treatment of radiation therapy or chemotherapy [28].

MMMT accounts for only 1.2 % of all EC, and the 5-year survival ranges from 65 % for stage I to 26 % for stage IV disease [29]. They contain both carcinomatous (epithelial) and sarcomatous (mesenchymal) elements. The carcinomatous component (CC) is endometrioid, serous, or clear cell, and the sarcomatous component (SC) is leiomyosarcoma, fibrosarcoma, endometrial stromal sarcoma, or heterologous [30]. There are two major theories for the origin of the biphasic nature of these tumors: a collision theory and monoclonal theory. In the collision theory, the two malignancies (epithelial and mesenchymal) arise separately and converge, whereas

the monoclonal theory purports that both components have the same origin but undergo divergent differentiation. Molecular studies to date have shown that these tumors are monoclonal and are most consistent with high-grade carcinomas with sarcomatous differentiation [30].

Diagnostic Dilemmas

1. Mixed tumors are usually composed of 2–3 different histopathological subtypes of EC. Most commonly, a low-grade endometrioid component is admixed with a high-grade component such as serous, clear cell, or grade 3 endometrioid. Clinically, a mixed tumor with 5–10 % of high-grade pathology is treated as a high-risk malignancy given that the clinical outcomes are similar to a pure type II tumor [31].
2. Complex atypical hyperplasia (CAH) is a precursor lesion of endometrioid endometrial carcinoma. There is high interobserver variability in the diagnosis of CAH. The study by Trimble and colleagues prospectively collected 306 patients with CAH who were diagnosed in community hospitals [32]. In these cases, up to 29 % of patients were upgraded to cancer upon re-review of the biopsy. Among these patients, 42.6 % had concurrent cancer on the hysterectomy specimen with 31 % showing myometrial invasion and 11 % with deep myometrial invasion. In 5.5 % of the cases, there was no consensus on the biopsy diagnosis, and 62.5 % of these had carcinoma in their hysterectomy specimens. The take-home point from this study was that the diagnosis of CAH/EEC can have high interobserver variability. In addition, up to 40 % of patients diagnosed with CAH have an underlying malignancy. In most clinical practices, CAH is treated as EEC, and intraoperative frozen section pathology is used to determine the extent of surgical staging needed in these patients.

Prognostic Factors

Based on clinicopathologic studies, several prognostic factors have been developed for EC. These include age at diagnosis of EC, size of tumor, depth of myometrial invasion, the presence or absence of lymphovascular space invasion, tumor histology including grade, involvement of the lower uterine segment, the presence of hormone receptors, lymph node metastases, adnexal metastases, and tumor stage [33, 34]. Women >age 65 have a worse overall survival than younger women. In part, this is related to the fact that EC is more commonly diagnosed in older women, especially type II carcinomas (Table 2.2). A classic clinicopathologic study of over 600 stage I EC by the Gynecologic Oncology Group 33 (GOG-33) established the relationship between tumor grade, depth of myometrial invasion, lymph node metastases, and overall tumor stage [34]. Overall, with the higher tumor grade, there

Table 2.2 Poor prognostic factors for endometrial carcinoma

Prognostic factor
Age > 65
Tumor size > 2 cm
The presence of lymphovascular space invasion
Grade 3, serous, clear cell, MMMT pathology
Myometrial invasion ≥ 50 %
The presence of tumor in the lower uterine segment
The absence of estrogen and/or progesterone receptor on tumor cells
Adnexal involvement
Lymph node involvement
High-surgical stage

Table 2.3 Risk of lymph node metastases in endometrial cancer

Tumor grade	# of patients	Depth of myometrial invasion			
		None	Inner 1/3rd	Middle 1/3rd	Outer 1/3rd
		% pelvic lymph node metastasis			
1	180	0	3	0	11
2	288	3	5	9	19
3	153	0	9	4	34
		% Periaortic lymph node metastases			
1	180	0	1	5	6
2	288	3	4	0	14
3	153	0	4	0	24

Adapted from [34]

was deeper myometrial invasion and increased extrauterine disease as well as lymph node metastases. Noninvasive or <30 % myometrial invasion was found in 77 %, grade 1 (G1); 56 %, grade 2 (G2); and 42 %, grade 3 (G3) tumors. Deep or >30 % myometrial invasion was found in 22 %, G1; 44 %, G2; and 58 %, G3 tumors. Similarly, higher-grade and deeper myometrial invasion were associated with pelvic and/or periaortic lymph node metastases (Table 2.3). The Mayo Clinic has also developed a criterion which uses tumor grade and intraoperative tumor size and depth of myometrial invasion to determine the extent of surgical staging. The group studied risk factors for pelvic lymph node metastases in 328 patients with low-grade endometrioid cancer with <50 % myometrial invasion who were treated surgically [35]. Pelvic lymphadenectomy was performed in 187 cases (57 %), and nodes were positive in 9 cases (5 %). The 5-year overall recurrence-free survivals were 97 % (lymphadenectomy) and 96 % (no lymphadenectomy), respectively. They concluded that patients who have grade 1 or 2 EC with greatest surface dimension ≤2 cm, myometrial invasion ≤50 %, and no intraoperative evidence of macroscopic disease can be treated optimally with hysterectomy only.

Peritoneal Cytology

While the FIGO guidelines recommend collection of pelvic cytology at the beginning of surgery, the results are no longer used in the staging system because they do not affect overall prognosis. A 2009 systematic review that included over 50 studies reported that the prognosis associated with a positive peritoneal cytology varied according to the presence of other factors [36]. Women with positive peritoneal cytology, but otherwise low-risk disease (grade 1 or 2, myometrial invasion <50 %, no cervical involvement, no lymphovascular space invasion), had a significantly lower rate of recurrence compared with other women (4.1 versus 32 %). The high-risk group includes those with grade 3 disease, clear cell or serous histology, deep myometrial invasion, or the presence of lymphovascular space invasion [37]. In these patients, adjuvant treatment is recommended based on uterine risk factors and stage, not peritoneal cytology alone.

Survival

As compared to type I endometrial cancer, stage for stage, the survival rates are 20–30 % less for type II EC [25]. Among women diagnosed with stage I serous, clear cell, or endometrioid cancers, the 5-year survival rate is 74, 88, and 95 %, respectively. Among women with stage II cancers, it was 56, 67, and 86 %, respectively. Among women with stage III cancers, it was 33, 48, and 67 %, respectively. Among women with stage IV cancers, it was 18, 18, and 37 %, respectively.

Initial Treatment of Endometrial Cancer

Surgical Management

All endometrial cancers are initially treated with surgical management. This includes a total hysterectomy, bilateral salpingo-oophorectomy, peritoneal cytology, and, if indicated, systematic pelvic and para-aortic lymphadenectomy. The role of the surgical management is treatment, evaluation of pathologic risk factors, and establishment of disease stage. For the majority of EC patients who have low-risk disease, surgical management is the only treatment required. The evaluation of pathologic risk factors and disease stage places the patients in four risk categories which determine the role of adjuvant treatment: low risk, intermediate risk, high-intermediate risk, and high risk (early and late stages). These will be discussed in more detail in the subsequent sections.

Minimally Invasive Surgery

The initial surgical approach can be abdominal laparotomy or a minimally invasive procedure. A complete vaginal approach is not recommended because the evaluation of the peritoneal cavity is required. Therefore, a vaginal hysterectomy can be combined with laparoscopy or robotic surgery to perform the cytology and lymph node dissection. A multi-institution study by the GOG established the safety of minimally invasive surgery for endometrial cancer staging [38]. Patients with clinical stage I to IIA uterine cancer were randomly assigned in a 2:1 ratio to laparoscopic staging or laparotomy to evaluate the study end points of a 6-week morbidity and mortality, hospital length of stay, conversion from laparoscopy to laparotomy, recurrence-free survival, site of recurrence, and patient-reported quality-of-life outcomes. Among the 1682 patients who were randomly assigned to laparoscopy, 74 % completed without conversion to laparotomy. Factors leading to conversion included poor visibility, findings of metastatic cancer, bleeding, and other less common causes. While laparoscopy had longer operative time, there were fewer moderate to severe postoperative adverse events and decreased hospitalization stay than laparotomy but similar rates of intraoperative complications. Fewer patients had pelvic and para-aortic node dissection with laparoscopy vs. laparotomy, which was mainly attributed to surgeon proficiency with laparoscopy. With a median follow-up time of 59 months, laparoscopy was not inferior to laparotomy. Among the 2181 patients still alive, there were 309 recurrences (210 laparoscopy; 99 laparotomy) and 350 deaths (229 laparoscopy; 121 laparotomy) [39]. The estimated 5-year overall survival was almost identical in both arms at 89.8 %. The minimally invasive surgical approach has translated to more patients having robotic surgery, which applies the same principles of laparoscopy. Operative time, hospitalization time, blood transfusion, and pain are improved with minimally invasive approach over laparotomy. Therefore, minimally invasive surgery in patients without evidence of peritoneal metastases is recommended.

Lymphadenectomy

The role of systematic pelvic and periaortic lymphadenectomy is more controversial. The GOG currently defines the boundaries of pelvic node dissection as removing nodal tissue from the distal half of each common iliac artery and anterior and medial tissue from the proximal half of the external iliac artery and vein as well as the distal half of the obturator fat pad [40]. Aortic node dissection involves removal of nodal tissue inferior from the inferior mesenteric artery to the mid-common iliac artery. This is usually considered standard of care by gynecologic oncology surgeons. If preoperative imaging shows evidence of bulky nodal disease in any of these surgical beds, then removing those and verifying metastatic disease is adequate, and a complete lymphadenectomy is not necessary.

The argument against systematic lymphadenectomy in EC is due to lack of prospective data supporting a survival benefit of the procedure. An international,

randomized controlled trial designed to evaluate overall survival in early-stage EC assigned patients to standard surgery (TH and BSO, peritoneal washings, and palpation of para-aortic nodes) or standard surgery plus lymphadenectomy [41]. There was no evidence of benefit in terms of overall or recurrence-free survival for pelvic lymphadenectomy in women with early endometrial cancer. Even though this study included 1408 women, criticisms included the investigators' subjectivity of node palpation intraoperatively, the preoperative stratification into low and high risk, lack of patients with high-risk pathologies, and the large variability in surgical practices. Data suggests that even lymph nodes that are palpated to be normal by a surgeon have a high false-negative rate. In addition, lymphadenectomy allows pathologic diagnosis of staging, which is paramount in determining if a patient should receive adjuvant therapy.

Given the previous clinicopathologic GOG-33 study, preoperative tumor grade along with intraoperative frozen section diagnosis of tumor size and depth of invasion is often used to determine the extent of lymphadenectomy. Researchers at the Mayo Clinic defined low risk as grade 1 or 2 endometrioid type with myometrial invasion (MI) $\leq 50\%$ and primary tumor diameter (PTD) ≤ 2 cm [35, 42]. Lymphadenectomy was not performed in these patients, which accounted for 27 % of all subjects. Sixty-three (22 %) of 281 patients undergoing lymphadenectomy had lymph node metastases: both pelvic and para-aortic in 51 %, only pelvic in 33 %, and isolated to the para-aortic area in 16 %. They concluded that systematic lymphadenectomy, including pelvic and periaortic, benefitted all those who do not meet the low-risk criteria. This criterion is often used to make a clinical operative decision regarding the extent of lymphadenectomy.

Sentinel lymph node mapping in endometrial cancer is another promising surgical approach to further define the lymphatic beds most susceptible to cancer spread. This technique is investigational at the time of this publication.

Surgical Cytoreduction

The role of surgical cytoreduction, removal of all visible disease to a microscopic level, is also not determined in endometrial cancer. Data has been extrapolated in high-risk EC to that from ovarian cancer, especially serous tubal/ovarian cancer, where there is a survival benefit to maximal surgical effort followed by adjuvant systemic treatment. High-grade EC often presents with metastatic disease outside the pelvis. Studies have shown improved survival following optimal primary cytoreductive surgery [43]. In one of the largest series of patients with advanced stage USC, optimal cytoreduction, defined as ≤ 1 cm maximal diameter of the largest residual tumor nodule at completion of primary surgery, was associated with a median survival of 39 months, compared to 12 months in patients who underwent a suboptimal surgical effort ($p = 0.0001$) [44]. Maximal cytoreduction is considered the goal of initial surgical management for those with bulky intraperitoneal disease.

Adjuvant Treatment After Surgery

Many clinical studies have been devoted to evaluating the role of adjuvant therapy to improve progression-free and overall survival in endometrial cancer patients. The role of adjuvant treatment—chemotherapy and/or radiation—is best defined if patients are categorized into risk groups for recurrence. These include the following: low, intermediate, high-intermediate, and high risk (Table 2.4). There is some overlap in the patients included in these groups.

Patients with low-risk disease, i.e., grade 1 or 2 tumors, no myometrial invasion, and lack of high-risk histologies (serous, clear cell, MMMT) have an extremely low risk of recurrence. No adjuvant treatment is recommended to patients in this group after surgery. Some of these patients who may desire fertility can be treated with progestin therapy, as will be discussed in more detail later in this chapter.

Intermediate-risk patients are defined as those whose cancer is confined to the uterus but invades the myometrium or has cervical stromal invasion [34, 40, 45, 46]. Type II cancers are excluded. Patients with FIGO stage IAG1, IAG2, IBG1, IBG2, and IAG3 and stage II will fall into this category. The risk of local recurrence is <10 %, and distant recurrence is negligible. Other adverse prognostic factors are used to stratify women with intermediate-risk endometrial cancer into low- or high-intermediate risks. These include the outer third of the myometrial invasion, grade 2 or 3 differentiation, or the presence of lymphovascular invasion within the cancer [34]. Low-intermediate risk patients are usually not recommended to have adjuvant treatment, while high-intermediate-risk patients will be discussed next. In a 2012 meta-analysis of eight trials that evaluated adjuvant radiation therapy (RT) for stage I endometrial cancer, among 517 women with low-risk disease, pelvic external beam radiation therapy (EBRT) was associated with increased risk of death related to endometrial cancer, secondary cancers, and treatment-related complications compared to observation [47].

Table 2.4 Definitions of endometrial cancer risk group after primary surgical management

Risk group	Definition	Recommended treatment after surgery
Low risk	Grade 1 or 2 with no myometrial invasion	Observation
Intermediate risk	IAG1, IAG2, IBG1, IBG2, and IAG3, stage II with <50 % myometrial invasion	Observation, consider VBT for high-risk patients
High-intermediate risk	Age > 60 with IBG1, IBG2, IAG3 with lymphovascular space invasion, IBG3, stage II with ≥50 % myometrial invasion	VBT, consider EBRT for the highest-risk patients
High-risk early stage	IBG3 with lymphovascular space invasion, all stage 1 and II uterine serous, clear cell, MMMT	Chemotherapy with or without EBRT/VBT
High-risk advanced stage	Stage III–IV, any pathology	Combination chemotherapy and EBRT/VBT

Adapted from [40, 45, 46]
MMMT malignant mixed mullerian tumor, *VBT* vaginal cuff brachytherapy, *EBRT* external beam pelvic radiation, *G* grade

Adjuvant Radiation Therapy

Several large US-based and international trials of adjuvant radiation after surgical staging for endometrial cancer have helped define the high-intermediate and high-risk categories and developed the guidelines for recommendation of adjuvant pelvic radiation [40, 45, 46, 48]. PORTEC-1 was a European randomized controlled trial of no further treatment or 46 Gy (EBRT) to women with stage 1 endometrial carcinoma (grade 1 with ≥50 myometrial invasion, grade 2 with any invasion, or grade 3 with superficial <50 % invasion) [45]. The primary study end points were local (vaginal, pelvic, or both) recurrence, and death, with treatment-related morbidity and survival after relapse as secondary end points. Among the 715 patients, randomized, local recurrences were diagnosed in 11 patients assigned to EBRT and in 40 assigned to observation. Five-year locoregional recurrence rates were 4 % in the radiotherapy group and 14 % in the control group; 73 % of these were vaginal-only recurrences. The overall incidence of distant metastases (peritoneum, lung, or both) was similar in the treatment groups: 8 % in the EBRT group and 7 % in the control group. From this study, patients at highest risk of recurrence were those >60 years of age, >2/3rd myometrial invasion, and grade 3 disease. Of note, this study excluded patients with deep myometrial invasion and grade 3 tumors. A subsequently published subset analysis of this study showed that actuarial 5-year rates of locoregional relapse were 14 % for those with deep myometrial invasion (outer third) and grade 3 disease [49]. Five-year distant metastasis rates were 3–8 % for grades 1 and 2 tumors; 20 % for <50 % MI, grade 3 tumors; and 31 % for outer third MI, grade 3 tumors. Overall survival rates were 83–85 %, 74 %, and 58 %, respectively. One of the major criticisms of this study is that because a systematic lymphadenectomy was not performed, patients with potential stage IIIC disease were missed and not given adjuvant chemotherapy. Regardless, this study reinforces the importance of uterine factors and tumor grade in determining the risk of recurrent disease.

A follow-up study, PORTEC-2, further randomized high-intermediate-risk patients to receive adjuvant 46 Gy EBRT or vaginal cuff brachytherapy (VBT; 21Gy high-dose rate or 30 Gy low-dose rate) only [46]. High-intermediate-risk patients were defined as follows: (1) age > 60 years, stage IB and grade 1 or 2 or stage 1A (<50 % MI) grade 3 disease; (2) stage 2A disease, any age. Grade 3 patients with greater than 50 % myometrial invasion were excluded, like in PORTEC-1. At median follow-up of 45 months, three vaginal recurrences were diagnosed after VBT and four after EBRT. Estimated 5-year rates of vaginal recurrence were 1.8 % VBT and 1.6 % for EBRT. There was no difference in overall survival rates of distant metastases or progression-free survival. Gastrointestinal toxicity was significantly less in those who received VBT vs. EBRT.

The GOG also performed a randomized controlled trial of surgery followed by observation or EBRT in patients with intermediate- and high-intermediate-risk disease (GOG-99) [40]. Based on GOG-33 data, they included all women found to have any degree of myometrial invasion with adenocarcinoma of any grade and no evidence of lymph node involvement on stages I and II. In the previous study, these patients were found to have most of the recurrences within 2 years after cancer

diagnosis. This study was plagued by enrolling too many patients with truly low-risk disease. Local recurrence rates were 8.9 % in observation vs. 1.6 % in EBRT group. Distant metastases were similar in both groups, 6.4 % vs. 5.3 %, respectively. Based on ad hoc analysis of age and three pathologic factors (deep MI, grade 2 or 3 pathology, or the presence of lymphovascular space invasion), high-intermediate-risk group patients were defined as follows: (1) ≥70 years with one risk factor, (2) age 50–69 years with two risk factors, or (3) age ≥18 years with all three risk factors. In the GOG-99 trial, two-thirds of all recurrences were in women who met these pathologic criteria.

Adjuvant Chemotherapy With or Without EBRT/VBT

The last category, high-risk patients, include all patients with any stage of USC, UCC, and MMMT; all stage III or IV endometrioid adenocarcinoma; and all patients with grade 3 endometrioid adenocarcinoma with deep myometrial invasion (>50 %). Based on PORTEC data, the IBG3 endometrioid group with lymphovascular space invasion had 14 % local recurrences and 31 % distant metastases. None of these patients were included in the previously discussed trial of adjuvant radiation therapy. In high-risk patients, combination of chemotherapy and radiation is usually recommended based on phase II and III clinical trial data.

High-Risk, Early-Stage EC

High-risk early-stage EC patients include those with (1) stage IB or II endometrioid grade 3 pathology, deep myometrial invasion (>50 % MI with highest risk being in those with outer third MI), and lymphovascular space invasion and (2) all stage I and II USC, UCC, and MMMT patients. Data supporting chemotherapy in addition to RT is based on small trials [50–52]. RTOG 9708 was a phase II trial that assessed the patterns of recurrence and survival when chemotherapy was combined with adjuvant radiation for patients with high-risk endometrial cancer (stages IBG2, IBG3, stage II or IIIC1) [50]. Patients received 45 Gy EBRT/VBT with concurrent cisplatin (50 mg/m^2) on days 1 and 28 followed by four cycles of cisplatin (50 mg/m^2) and paclitaxel (175 mg/m^2) given at 4-week intervals following completion of radiotherapy. In a total of 46 patients, pelvic, regional, and distant recurrence rates were 2 %, 2 %, and 19 %, respectively. Overall survival and disease-free survival (DFS) rates at 4 years are 85 % and 81 %, respectively. Four-year rates for survival and DFS for stage III patients are 77 % and 72 %, respectively. None of the patients with stages IC, IIA, or IIB recurred. The treatment was overall well tolerated with 16 %, grade 1; 41 %, grade 2; 16 %, grade 3; and 5 %, grade 4. Sixty percent of the enrolled patients had stage IIIC1 disease. Therefore, it is difficult to tease out the effect for high-risk, early-stage patients.

A Japanese phase III trial randomized stage IB-IIIC1 patients with >50 % MI to adjuvant EBRT (40Gy) vs. cyclophosphamide-doxorubicin-cisplatin (CAP) [51].

The 5-year progression-free survival rate in the EBRT and CAP groups were 83.5 % and 81.8 %, respectively; OS rates were 85.3 % and 86.7 %, respectively. Among 120 patients in a high- to intermediate-risk group defined as [1] stage IB in patients over 70 years old or with G3 endometrioid adenocarcinoma or [2] stage II or IIIA (positive cytology), the CAP group had a significantly higher PFS rate (83.8 % versus 66.2 %, $P = 0.024$). There were no treatment-related deaths. EBRT toxicity was mainly in the chemotherapy group and consisted of bowel obstruction and myelosuppression.

A third phase II trial included surgically staged I–II EC patients who met GOG-99 high-risk criteria and stages I–II serous and clear cell cancers [52]. Patients were treated with 21 Gy VBT followed by three cycles of carboplatin (AUC 6) and paclitaxel (175 mg/m^2) chemotherapy. The study enrolled 23 patients, of whom 83 % completed the entire regimen. With a median follow-up less than 4 years, 91 % of patients remained disease free. Four patients experienced local and distant recurrences.

The GOG recently completed enrollment in randomized phase III study with a population similar to the last study in which patients were randomized to RT vs. chemotherapy and RT. The results are pending. As is evident, data supporting the use of chemotherapy and RT as adjunct treatments in the early-stage high-risk EC patients is heterogeneous and based on small clinical trials. It will be important to see if the cooperative group trials start enrolling patients based on their molecular profile more than histopathology and stage only.

Stages III–IV EC

The benefit of adjuvant chemotherapy for women with stage III endometrial cancer is supported by meta-analysis that included the data from two GOG randomized trials ($n = 620$) [53]. Compared with RT alone, the administration of platinum-based combination chemotherapy resulted in improvements in overall and progression-free survival [29]. Choices of chemotherapy regimen include carboplatin-paclitaxel (CP) or cisplatin-doxorubicin-paclitaxel-filgrastim (TAP). These trials are discussed in more detail in the recurrent disease section as all of these trials included patients with new diagnosis of stage III disease or chemotherapy-naïve recurrent disease. RT is often added to chemotherapy in stage III disease but does increase the risk of acute (e.g., myelosuppression) and late toxicities (e.g., radiation enteritis) and has not been proven to extend survival in this setting. Data indicating the feasibility and improved outcomes of combined-modality treatments comes from small phase II or retrospective studies [54, 55]. The final decision is usually left to the discretion of the patient, her gynecologic oncologist, and the radiation oncology specialist. If both chemotherapy and radiation are administered, VBT can be given concurrently with chemotherapy, and the EBRT can be given in the beginning, middle ("sandwich"), or end of chemotherapy [54, 56].

Multimodality therapy is typically recommended and highly individualized for USC, UCC, and MMMT given the aggressive course of these tumors. In patients with serous carcinoma, those with no residual carcinoma in the final hysterectomy

specimen, disease confined to a polyp or the endometrium without any myometrial invasion may be offered observation or VBT alone [57]. For all other patients, chemotherapy with (or without) tumor-directed RT is the preferred option [58, 59]. Adjuvant carboplatin-paclitaxel therapy improves survival in patients with uterine serous adenocarcinoma and clear cell adenocarcinoma, whereas ifosfamide/paclitaxel is recommended for MMMT [55, 58–61]. Whole abdominopelvic RT is no longer recommended because chemotherapy with (or without EBRT/VBP) appears to be more effective [62].

For uterine MMMT, ifosfamide-paclitaxel combination therapy increased survival and was less toxic than the previously used cisplatin/ifosfamide regimen [56, 60, 61, 63]. Overall survival was 13.5 months with ifosfamide/paclitaxel vs. 8.4 months with ifosfamide alone. However, the toxicity of ifosfamide has led to investigation of better-tolerated regimens. A phase II trial suggests that paclitaxel/carboplatin is also a useful regimen for carcinosarcoma. Adjuvant pelvic RT also decreases the rate of local recurrences when compared with surgery alone [64].

Two phase II studies by the same group have shown the feasibility and efficacy of the so-called "sandwich" treatment [55, 56]. Pelvic radiation therapy is "sandwiched" between chemotherapy in the following manner: three cycles of chemotherapy-EBRT/VBP-three cycles of chemotherapy. Of the 81 USC patients enrolled, 80 % completed the entire course of EBRT/VBT with three cycles of CP before and after. In the MMMT study, 70 % completed RT sandwiched between either ifosfamide or ifosfamide-cisplatin chemotherapy. Given the toxicity profile of these regimens, this is the preferred modality in patients with USC, CC, or MMMT and no residual disease after surgical staging.

Other Primary Treatment Modalities

Other treatment modalities that are current areas of research include the addition of metformin to the primary treatment of chemotherapy-naïve endometrial cancer patients. Metformin has received much press due to its anticancer potential. The exact mechanism is not known but may be related to the mTOR pathway, which has been implicated in endometrial cancer pathogenesis. Metformin's downstream target is AMP-activated protein kinase (AMPK), and its activation leads to regulation of multiple signaling pathways involved in the control of cellular proliferation, including inhibition of the mammalian target of rapamycin (mTOR) pathway. Preclinical data finds that metformin is a potent inhibitor of cell proliferation in endometrial cancer cell lines and that this effect is partially mediated through inhibition of the mTOR pathway [65]. In addition, treatment with metformin in combination with paclitaxel results in a synergistic antiproliferative effect in these cell lines [66]. Thus, metformin may have important therapeutic implications for EC.

Small case series also suggest a role of neoadjuvant chemotherapy prior to any surgical management in patients with stage IV, especially serous type. Some of these patients are not surgical candidates at the time of initial diagnosis and may benefit with initial chemotherapy to decrease the disease burden followed by surgery. Data supporting this is very limited [43, 67, 68].

Fertility-Sparing Treatments

Up to 15 % of women diagnosed with EC are less than 45 years of age [9]. Many of these patients have conditions that predispose them to excess estrogen (e.g., chronic anovulation, diabetes, and obesity or a genetic predisposition). Premenopausal women are more likely to develop type I endometrioid endometrial cancers, with early-stage disease [69, 70]. Fertility preservation is an important consideration in these patients. Patient selection for conservative management is important and includes those who desire and are planning fertility in a short period of time; have endometrial confined disease confirmed by a pelvic MRI, grade 1 disease; and those patients who are going to be compliant with the therapy. Options for these include preservation of the ovaries at the time of hysterectomy or primary management of the CAH or stage I EC with hormonal therapy.

Data to support ovarian preservation comes from studies indicating the safety of estrogen replacement therapy in patients with low-risk endometrial cancer after TAH-BSO and from population-based studies of young women with low-risk disease. Two large prospective trials of hormone replacement therapy after TAH-BSO for endometrial carcinoma showed no marked increase in recurrence risk. In a randomized, placebo-controlled, double-blind trial by the GOG, the absolute recurrence rate in the estrogen-treated arm was 2.1 % compared to 1.9 % in the placebo arm [71]. In a second prospective cohort study of 102 patients, combination estrogen and progesterone therapy produced no increased risk of recurrence over control patients [72].

Ovarian preservation is supported by multiple population-based studies that show no difference in overall survival or recurrence in young women with low-risk disease. In a study by Lee and colleagues of 260 patients who underwent surgical treatment, 19 (7.3 %) had ovarian tumors: 12 were metastatic endometrial and 7 were synchronous ovarian primary cancer [73]. Intraoperative extrauterine disease was the most significant predictor of ovarian involvement and was present in 17 of the 19 patients with ovarian involvement. Of note, there were no cases of ovarian involvement in the subset of patients younger than 45 years with no intraoperative evidence of extrauterine disease. These findings contrast to another review in which four patients (all younger than 45 years) had normal intraoperative findings and diagnosis of ovarian involvement on final pathology [74]. A Surveillance, Epidemiology, and End Result (SEER) population study of 3269 women with endometrial cancer showed that ovarian conservation did not have any detrimental effects on survival in patients younger than 45 years with stage I cancers [75]. Overall survival was 98 % for patients with stage IA non-myoinvasive disease, regardless of oophorectomy. For patients with stage IB EC, survival was 86 % in those who had oophorectomy and 89 % for those with ovarian preservation. Furthermore, in a Korean retrospective study of 175 patients who had undergone ovarian preservation, recurrence-free survival and overall survival rates were 94.3 % and 93.3 %, respectively [76]. None of the seven documented recurrences occurred in stage I patients with low-grade, non-myoinvasive disease.

Therefore, patients less than 45 years of age who desire fertility and meet other low-risk criteria can be offered medical management or ovarian preservation under the guidance of a gynecologic oncologist. It is important to evaluate the adnexa in

these women to rule out ovarian disease. Patients can be treated with high doses of progesterone (medroxyprogesterone acetate or megestrol acetate) orally and/or levonorgestrel intrauterine device [77, 78]. There are no randomized controlled data indicating superiority of one regimen over the other. Most of the progestin studies are small, retrospective reviews of oral progestins. Overall response rates to progestin therapy are 50–80 % complete response within 12 weeks of initiating therapy. Risk factors for lack of response include BMI > 35 or lack of pathologic treatment response (exogenous progestin effect) after 3 months [78]. Up to 20 % of these patients will experience disease relapse and close monitoring even after they have completed childbearing is important. Recent data indicate that use of assisted reproductive technologies is safe for these patients in achieving pregnancy in a timely manner. The success rates of in vitro fertilization in this group of patients can be 30 %, which is equivalent to national IVF success rates [77].

Primary Radiation Therapy

Primary radiotherapy in lieu of surgery can be used in certain circumstances. Some patients are not candidates for surgery. These include patients with severe comorbidities that limit administration of anesthesia, such as severe cardiac disease, chronic obstructive pulmonary disease, pulmonary hypertension, and others. In other patients with advanced disease and overall poor life prognosis due to age or comorbidities, primary radiation can be recommended as a palliative measure to stop pelvic bleeding and reduce the risk of a fatal hemorrhage. Finally, patients with locally advanced disease involving the parametria or vagina can be recommended for low-dose radiation to shrink the tumor and allow a subsequent surgical effort. Injuries to the genitourinary tract and/or colon are more common in these patients given the proximity of these organs and damage and poor healing from radiation [79].

Surveillance After Primary Treatment

There is no demonstrated value of intensive surveillance in endometrial cancer. Most patients are recommended to see their physician for a pelvic and physical exam every 3–6 months for 3–5 years after the initial diagnosis [64]. The use of vaginal cytology is no longer recommended for asymptomatic patients consistent with the Society of Gynecologic Oncology guidelines [80]. Patients with stage I endometrial cancer have a low risk of asymptomatic vaginal recurrence (2.6 %), especially after adjuvant brachytherapy, and vaginal cytology is not independently useful for detecting recurrences in this group of patients. Patients with clinical stage I and stage II endometrial cancer have a recurrence rate of approximately 15 % within 3 years of initial treatment [81, 82]. Because most recurrences are symptomatic, patients are counseled regarding the symptoms of recurrent disease including

bleeding (vaginal, bladder, or rectal), decreased appetite, weight loss, pain (in the pelvis, abdomen, hip, or back), cough, shortness of breath, and swelling (in the abdomen or legs). Imaging can be performed as clinically indicated if patients present with any symptoms suggestive of disease recurrence.

An exception to routine imaging may be those patients with initially advanced disease such as stages III–IV or high-risk histopathologies such as serous, clear cell, or MMMT tumors. In these patients, annual imaging with CT for 5–10 years has been recommended to evaluate for recurrent disease. Given that most recurrences are symptomatic and treatment for recurrent disease is limited, some clinicians do not advocate for intense monitoring with imaging [83].

Serum biomarkers for endometrial cancer have also been evaluated. Cancer antigen 125 or CA-125 is the most studied biomarker for endometrial cancer. Multiple studies support the use of CA125 as a marker of extrauterine disease and for surveillance for recurrent disease in patients with uterine cancer [84, 85]. Olawaiye et al. analyzed the outcomes of 41 patients with USC who had preoperative CA125 measurement [85]. They reported that preoperative CA125 levels correlated with disease stage. In addition, CA125 elevation was adversely associated with survival in multivariate analysis. In another study, in multivariate survival analysis, an elevated CA125 level compared to non-elevated CA125 was not associated with disease recurrence [84]. There is no recommendation to routinely screen endometrial cancer patients for recurrence with serum CA125.

Survivorship Issues in Endometrial Cancer Patients

In the absence of recurrence, posttreatment surveillance provides psychosocial reassurance and improves the quality of life for patients and their families. Survivorship issues were taken precedent for patients, many of whom have long-term survival and associated medical comorbidities. Patients are counseled to have routine cancer screening for breast and colon cancers. There is a recent focus on management of obesity and cardiovascular health as many endometrial cancer patients are obese. Some groups have advocated referring patients aggressively for bariatric surgery or other weight loss programs. Long-term survival data indicate that many endometrial cancer patients eventually die of complications of cardiovascular disease [80].

Toxicity related to adjuvant chemotherapy and radiation therapy must be addressed [86]. These include peripheral neuropathy, fatigue, chronic anemia, menopausal symptoms, lymphedema, sexual dysfunction, and gastrointestinal toxicity, among others. Peripheral neuropathy is usually a sequela of chemotherapy management with carboplatin and paclitaxel. While it's often irreversible, it can be managed symptomatically with duloxetine, gabapentin, or other supportive therapies. Chronic fatigue is commonly seen in patients after chemotherapy. A multidisciplinary approach to this is important to coordinate psychosocial factors and to evaluate for depression. As discussed earlier, while hormonal therapy

is not prescribed routinely, young women or those with severe vasomotor symptoms after TAH/BSO may benefit from short course of estrogen therapy without increasing the risk of disease recurrence. Lymphedema, often seen in those who had adjunct radiation therapy, is treated with supportive care and physical therapy. Radiation-associated gastrointestinal toxicity can be mild and treated with dietary changes to a severe protein-losing enteropathy that requires nutritional supplementation. Comanagement with a gastroenterologist is usually required for these patients. Patients should be educated regarding sexual health, vaginal dilator use, and vaginal lubricants or moisturizers. Sexual dysfunction can be a sequela of surgical menopause and pelvic and intravaginal radiation therapy.

Treatment of Recurrent Disease

Even though overall deaths associated with endometrial cancer are low compared to other cancers that affect women, treatment of recurrent disease is limited especially in those who have received prior adjuvant chemotherapy and/or radiation. Imaging with CT or PET/CT is initially performed to determine the extent of recurrent disease. Most of the disease failures can be characterized as local failure (pelvic or vaginal recurrence) or systemic metastatic disease. Most recurrences are treated with radiation and/or chemotherapy, hormonal therapy, or palliative measures. The role of surgical cytoreduction is limited given the efficacy of radiation in localized disease and lack of efficacy of surgery in disseminated disease. Some patients do require palliative surgical measures such as intestinal diversion for blockage or gastrointestinal tubes for venting.

Localized Recurrence

Patients with local or regional recurrences can be evaluated for radiation treatment. For patients with no prior RT exposure at the recurrence site or previous brachytherapy only, RT plus brachytherapy is recommended. Isolated vaginal recurrences treated with RT have good local control and 5-year survival rates of 50–70% [87, 88]. Prognosis is worse if there is an extravaginal extension or a pelvic lymph node involvement [88]. After RT, it is unusual for patients to have recurrences confined to the pelvis. The management of such patients remains controversial. For patients previously treated with EBRT at the recurrence site, recommended therapy for isolated relapse includes surgery with (or without) intraoperative RT (IORT), hormonal therapy, or chemotherapy. Radical surgery, such as pelvic exenteration, in highly selected patients with central pelvic recurrence in the radiated field has been performed with reported 5-year survival rates approximating 20% [89, 90].

Systemic Disease: Hormonal Therapy

The role of hormonal therapy in recurrent or metastatic cancer has been primarily evaluated in patients with endometrioid histologies only. Progestational agents, tamoxifen with alternating megestrol, and aromatase inhibitors may be used [91–93]. No particular drug, dose, or schedule has been found to be superior. The main predictors of response in the treatment of metastatic disease are well-differentiated tumors, expression of ER/PR receptors, a long disease-free interval, and the location and extent of extrapelvic (particularly pulmonary) metastases [91]. For asymptomatic or low-grade disseminated metastases, hormonal therapy with progestational agents has shown good responses, particularly in patients with ER/PR-positive disease [94–96]. Tamoxifen has a 20 % response rate in those who do not respond to standard progesterone therapy [97]. Tamoxifen has also been combined with progestational agents but its use is limited by higher incidence of thromboembolic events. If disease progression is observed after hormonal therapy, cytotoxic chemotherapy can be considered. However, clinical trials or best supportive care are appropriate for patients with disseminated metastatic recurrence who have a poor response to hormonal therapy and chemotherapy.

Systemic Disease: Chemotherapy

Based on the current data, multiagent chemotherapy regimens are preferred for metastatic, recurrent, or high-risk disease, if tolerated. In a phase III randomized trial (GOG 177), women with advanced/metastatic or recurrent endometrial carcinoma were randomly assigned to two combination regimens: cisplatin, doxorubicin, and paclitaxel (TAP) or cisplatin and doxorubicin (AP) [98, 99]. Women who received TAP had an improved survival (15 versus 12 months, $P<0.04$) but with significantly increased toxicity (i.e., peripheral neuropathy). The use of TAP regimen is therefore limited by its toxicity. The response rates with other multiagent chemotherapy have ranged from 31 to 81 % but with relatively short durations. The median survival for patients in such trials remains approximately 1 year [100].

Carboplatin and paclitaxel (CP) is an increasingly used regimen for advanced/metastatic or recurrent endometrial cancer; the response rate is about 40–62 %, and overall survival is about 13–29 months [101, 102]. A phase III trial (GOG 209) compared CP vs. TAP. The final data are still maturing, but data presented at the 2015 Society of Gynecological Oncologists annual meeting showed that oncologic outcomes are similar, but the toxicity and tolerability profile favor CP.

If multiagent chemotherapy regimens are contraindicated, then single-agent therapy options include paclitaxel, cisplatin, carboplatin, doxorubicin, liposomal doxorubicin, topotecan, and docetaxel [94, 103]. When single agents are used as first-line treatment, responses range from 21 to 36 % [104]. When single agents are used as second-line treatment, responses range from 4 to 27 %; paclitaxel is the

most active in this setting [104]. Liposomal doxorubicin is commonly used because it is less toxic than doxorubicin, but the response rate of liposomal doxorubicin is low at 9.5 % [105]. New biologic and molecular therapies for the treatment of recurrent or metastatic endometrial carcinoma are being assessed in clinical trials (see Chap. 6) [106]. Bevacizumab was shown to have a 13.5 % response rate and overall survival rate of 10.5 months in a phase II trial for persistent or recurrent endometrial cancer. Temsirolimus has been used as first-line or second-line therapy for recurrent or metastatic endometrial cancer and has a partial response rate of 4 % in second-line therapy [107]. Other agents, such as PI3kinase inhibitors, are currently in early-stage development; their use may be limited by additional toxicity such as hyperglycemia and mood changes [108, 109]. Clinical trials evaluating new cytotoxic therapies and targeted agents in endometrial cancer are ongoing.

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Chapter 3
Pathology of Endometrial Carcinoma

Sigurd F. Lax

Abstract On a clinicopathological and molecular level, two distinctive types of endometrial carcinoma, type I and type II, can be distinguished. Endometrioid carcinoma, the typical type I carcinoma, seems to develop through an estrogen-driven "adenoma carcinoma" pathway from atypical endometrial hyperplasia/endometrioid intraepithelial neoplasia (AEH/EIN). It is associated with elevated serum estrogen and high body mass index and expresses estrogen and progesterone receptors. They are mostly low grade and show a favorable prognosis. A subset progresses into high-grade carcinoma which is accompanied by loss of receptor expression and accumulation of TP53 mutations and behaves poorly. Other frequently altered genes in type I carcinomas are K-Ras, PTEN, and ß-catenin. Another frequent feature of type I carcinomas is microsatellite instability mainly caused by methylation of the MLH1 promoter. In contrast, the typical type II carcinoma, serous carcinoma, is not estrogen related since it usually occurs in a small uterus with atrophic endometrium. It is often associated with a flat putative precursor lesion called serous endometrial intraepithelial carcinoma (SEIC). The molecular pathogenesis of serous carcinoma seems to be driven by TP53 mutations, which are present in SEIC. Other molecular changes in serous carcinoma detectable by immunohistochemistry involve cyclin E and p16. Since many of the aforementioned molecular changes can be demonstrated by immunohistochemistry, they are useful ancillary diagnostic tools and may further contribute to a future molecular classification of endometrial carcinoma as recently suggested based on The Cancer Genome Atlas (TCGA) data.

Keywords Endometrial carcinoma • Histopathology • Molecular pathways • Prognosis • Grading • Typing

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Introduction

Endometrial carcinoma is the most frequent neoplasm of the female reproductive organs in the industrialized countries with the highest incidence in North America and Europe. In 2008, 288,000 new cases were diagnosed worldwide, 40,000 of them in the USA. In the same year, about 7500 women died from endometrial carcinoma in the USA [1]. The incidence rate varies significantly throughout the world with clearly lower rates in developing countries but also Japan [2]. There is also a two-fold higher incidence in Caucasians compared to African Americans, but the latter seem to be affected by more aggressive tumor types [3]. These global differences in the incidence are not well understood, but there seems to be an influence of age and a so-called Western lifestyle with Western diet, high body mass index, and low physical activity [4, 5]. Unopposed estrogens play an important pathogenetic role in postmenopausal women [6].

The histopathological classification of endometrial carcinoma distinguishes between various types of tumors with distinctive microscopic features. The most recent classification proposed by the WHO is listed in Table 3.1 and will be further discussed in detail [7]. Recent molecular studies support the histological and biological differences between the major subtypes of endometrial carcinoma by demonstrating distinctive molecular genetic differences. A proposal for a pathogenetic model attempts to combine the histological classification with molecular findings [8–10].

Table 3.1 Histopathological classification of endometrial carcinoma

• Endometrioid adenocarcinoma, usual type
• Endometrioid adenocarcinoma, variant types
– With squamous differentiation
– With secretory differentiation
– Villoglandular
– With mucinous differentiation
– Ciliated cell type
• Mucinous carcinoma
• Serous endometrial intraepithelial carcinoma
• Serous adenocarcinoma
• Clear cell adenocarcinoma
• Neuroendocrine carcinoma
– Low-grade neuroendocrine tumor/carcinoid tumor
– High-grade neuroendocrine carcinoma
• Small cell neuroendocrine carcinoma
• Large cell neuroendocrine carcinoma
• Mixed carcinomas
• Undifferentiated carcinoma
• Dedifferentiated carcinoma

A Putative Pathogenetic Model for Endometrial Carcinoma

A simplified model has been developed based on clinicopathological and molecular parameters to better understand endometrial tumorigenesis. According to this model, two types of endometrial carcinomas, characterized by distinctive morphological features and different pathogenetic pathways, can be distinguished (Table 3.2). Type I carcinomas, which account for the great majority of endometrial carcinoma (approximately 80–90 %), are characterized by low stage at diagnosis and a favorable clinical course. They typically develop in a normal-sized or myohyperplastic uterus and are associated with disordered proliferative or hyperplastic endometrium. The latter reflects unopposed estrogenic stimulation, which may be caused by persistent follicles due to anovulatory cycles, an estrogen-producing tumor such as adult granulosa cell tumor, endogenous estrogen production by aromatase present in adipose tissue, or hormone replacement therapy by pure estrogens. Thus, the typical age of patients with type I carcinomas is within the peri- and postmenopausal period. The patients also show elevated levels of free estrogen in the serum. Histologically, the prototype of type I carcinoma is endometrioid carcinoma including its variants and mucinous carcinoma. The tumors usually demonstrate low histological grade (well or moderate differentiation). Atypical endometrial hyperplasia/endometrioid intraepithelial neoplasia (AEH/EIN) is considered the immediate precursor lesion. The fact that these carcinomas usually highly express estrogen (ER) and progesterone receptors (PR) further underlines their relationship to estrogen.

In contrast, type II carcinomas are diagnosed at high stage and show an aggressive behavior with poor outcome. The histological prototype is serous carcinoma, but it also includes clear cell, undifferentiated carcinomas and a subset of grade 3 endometrioid carcinomas. These tumors are typically not related to estrogens, which are reflected by the following features: They usually occur in an atrophic uterus and are associated with atrophic or inactive endometrium. They may also occur in atrophic polyps. Serum estrogen is low in these patients. In addition, type II carcinomas often exhibit low ER expression and often lack expression of PR or may be ER and PR negative. Serous endometrial intraepithelial carcinoma (SEIC)

Table 3.2 Two major types of endometrial carcinoma

Features	Type I carcinoma	Type II carcinoma
Age (median)	60	70
Serum estrogen	Elevated	Low
Adjacent endometrium	Hyperplastic/disordered proliferative	Atrophic
Uterus, myometrium	Enlarged or normal, myohyperplasia	Atrophic
Stage at diagnosis	Low	Frequently increased
Histological type	Endometrioid and variants	Serous
Precursor	Atypical hyperplasia/EIN	Serous EIC
Clinical course	Typically favorable	Typically poor
Molecular alterations	PTEN and K-Ras mutations, MSI	P53 mutations

has been considered the immediate precursor of serous carcinoma but is now considered noninvasive carcinoma since it is frequently associated with extensive extrauterine disease. In this setting SEIC may be part of extensive pelvic serous carcinoma without clear site of origin. For other type II carcinomas, putative precursors are unknown although SEIC has been found in a subset of endometrial clear cell carcinomas.

Type I and type II carcinomas are also distinct at the molecular level [9]. Most type I carcinomas are characterized by minor changes of the genome as determined by a low number of somatic copy number alterations, whereas most type II carcinomas are characterized by major changes in the genome such as a high number of somatic copy number alterations and aneuploidy. Among the involved genes frequently mutated in type I carcinomas are *PTEN* (>50 %), *KRAS* (20–30 %), *ARID1A* (40 % of low-grade endometrioid carcinomas), *CTNNB1* (ß-catenin) (30 %), and *PIK3R1* (20–45 %), whereas mutations of *TP53* (80–90 %), *FBXW7* (20–30 %), and *PPP2R1A* (20–30 %) are more frequently found in type II carcinomas [11–17]. In addition, a mutator phenotype leading to microsatellite instability (MSI) is found in 25–40 % of type I carcinomas but very rare in type II carcinomas (<5 %). Microsatellite instability leads to an increased mutation rate often involving repetitive sequences [18]. On the other hand, mutations of *PIK3CA* are found almost equally in type I and type II carcinomas [19–21]. In addition, *TP53* mutations are found in a subset of grade 3 endometrioid carcinomas (30 %) [14].

The studies of The Cancer Genome Atlas (TCGA) project revealed four prognostic groups of endometrial carcinoma of which tumors with "serous-like" genomic changes particularly high copy number changes showed the worst prognosis. Tumors with mutations in the polymerase E (*POLE*) gene showed an excellent prognosis; the prognosis of tumors with low copy number changes and of hypermutated tumors was in between [22]. Recent studies reported *POLE* mutations in endometrial carcinomas with an excellent prognosis showing a serous and high-grade endometrioid phenotype, respectively [23]. Subsequently, a novel molecular-based classification system for endometrial carcinoma has been proposed including immunohistochemistry for p53 and mismatch repair proteins as well as mutational analysis for *POLE* [24].

Although clear cell carcinomas are considered biologically and clinically type II carcinomas, they share some molecular alterations with type I carcinomas, in particular *PTEN* mutations (30–40 %) and loss of *ARID1A* expression without intragenic mutations (25 %) [25, 26]. A recent study found a serous-like mutation profile of clear cell carcinoma with concurrent mutations in *TP53* and *PPP2R1A* but wild-type *ARID1A*, *PTEN*, *CTNNB1*, and *POLE* [27].

In summary, type I carcinomas often arise from atypical hyperplasia/EIN and may progress from low-grade into high-grade carcinomas. Some of the molecular changes seem to occur early, particularly in atypical hyperplasia and grade 1 endometrioid carcinoma, respectively, such as mutations in *PTEN*, *KRAS*, and *ARID1A*; others seem to represent late events since they occur in high-grade endometrioid carcinomas such as *TP53* mutations [14, 15, 28]. In contrast, serous carcinomas seem to develop de novo from atrophic endometrium through SEIC [29]. Mutations

of *TP53, PIK3CA*, *FBXW7*, and *PPP2R1A* as well as overexpression of *Cyclin E1* are considered early events in the development of serous carcinomas since they are present in SEIC [17, 30, 31]. Some of these genetic alterations seem to be strong drivers of tumorigenesis. In particular, mutated *TP53* seems to be a strong driver for growth in serous carcinoma leading to a strong selective advantage. The diffuse strong or flat negative immunoreactivity, which accompanies *TP53* mutations, seems to reflect an early clonal expansion that involves the whole tumor.

Hereditary Endometrial Carcinoma

In particular, hereditary non-polypous colorectal cancer (HNPCC)/Lynch syndrome and Cowden syndrome are heritable syndromes associated with an increased risk for endometrial carcinoma [32, 33]. Lynch syndrome is characterized by germline mutations in the mismatch repair genes *MLH1*, *MSH2*, *MSH6*, or *PMS2* and is associated with carcinomas of the colon/rectum and the endometrium. In addition, transitional cell carcinomas of the urogenital tract and ovarian carcinomas may occur. Patients with Cowden syndrome harbor germline mutations in *PTEN* and may be affected by carcinomas of various organs such as the uterus (endometrium), the thyroid, and the breast. About 2 % of all endometrial carcinomas are associated with Lynch syndrome of which most are of endometrioid histology [34]. Recently, other histological types have been described in patients with Lynch syndrome, particularly the dedifferentiated variant of undifferentiated carcinoma. There is evidence that a subset of these tumors arise from the lower uterine segment. In Lynch syndrome there is a 20 and 60 % lifetime risk of developing atypical hyperplasia and endometrial carcinoma, respectively [33, 34]. Endometrial carcinoma may anticipate or follow the diagnosis of colorectal carcinoma. Late onset of either endometrial or colorectal carcinoma is not unusual for Lynch syndrome since the median age of diagnosis for both cancers is slightly above 60 years. Particularly due to small family size and late onset of disease, selection criteria for Lynch mutation carriers such as Amsterdam II and Bethesda II, respectively, are considered increasingly less reliable. Therefore, screening of all newly detected endometrial carcinomas by immunohistochemistry has recently been proposed [35].

Endometrioid Carcinoma

Endometrioid adenocarcinoma, which typically displays a glandular, papillary, or solid pattern, is the most frequent histological type of endometrial carcinoma [7, 36, 37]. The glandular structures are typically well formed and show regular luminal borders resembling the glands of nonneoplastic endometrium. The nuclei are elongated and pseudostratified or round. Villous and papillary structures are commonly found and need to be distinguished from the papillae of serous carcinoma.

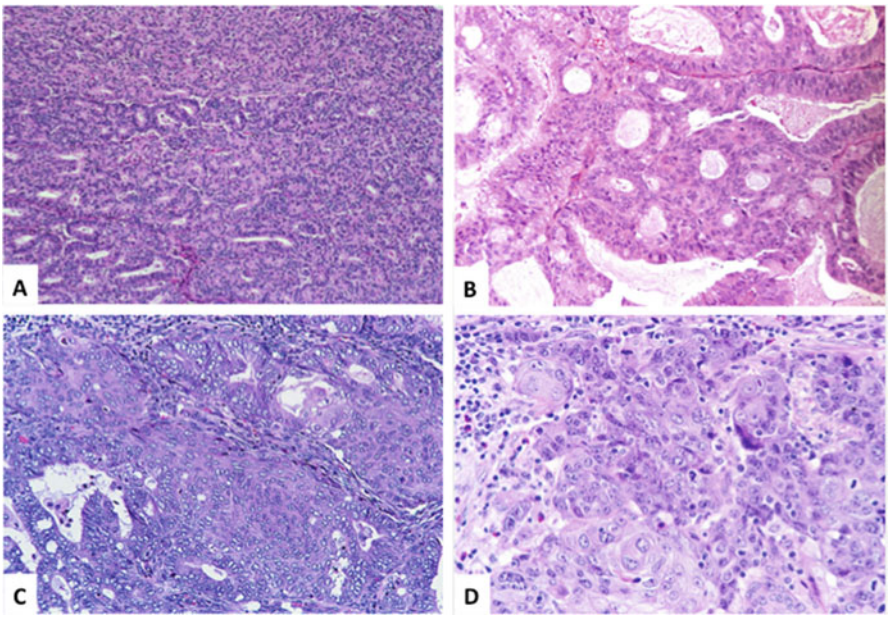

Fig. 3.1 Endometrioid carcinoma and variants: Moderately differentiated (FIGO grade 2) endometrioid carcinoma showing a mixture of glandular and solid structures (**A**). Well-differentiated (FIGO grade 1) endometrioid carcinoma with mucinous differentiation (**B**). Well-differentiated (FIGO grade 1) endometrioid carcinoma with squamous differentiation forming squamous morules (**C**). Poorly differentiated (FIGO grade 3) endometrioid carcinoma with squamous differentiation showing irregularly distributed atypical squamous nests (**D**)

The amount of solid non-squamous areas determines the histopathological grade of endometrioid carcinoma as determined by FIGO. In FIGO grade 1 carcinomas, solid areas account for less than 6 %, in FIGO grade 2 carcinomas 6–50 %, and FIGO grade 3 carcinomas more than 50 % of non-squamous solid areas (Fig. 3.1a, b). These solid areas need to be separated from areas of squamous differentiation, which are not considered for grading.

A subset of endometrioid carcinomas is associated with extensive lymphvascular space involvement (LVSI) which is considered a prognostic factor for recurrence but not predictive for lymph node metastases. An unusual pattern of tumor growth showing microcystic elongated and fragmented glands (MELF) seems to be frequently associated with LVSI [38]. Myometrial invasion may be clearly recognizable, particularly when it shows haphazardly distributed glands or diffusely arranged cords and clusters of cells or individual cells. The infiltrated myometrium frequently shows a desmoplastic reaction or less often an inflammatory response. On the other hand, myometrial invasion may appear smoothly showing pushing borders of the infiltrating tumor and a lack of desmoplasia. This pattern has been described as adenoma malignum-like [39]. A similar growth pattern is found when endometrial carcinoma extends into adenomyosis. The distinction from true myometrial invasion

is important since prognosis is not adversely influenced. This distinction may be difficult, particularly when glands in adenomyosis are sparse and the stroma is atrophic. Thus, the presence of clearly recognizable adenomyosis on H&E sections is required for the diagnosis of carcinoma involving adenomyosis. The diagnosis of superficial myometrial invasion can also be problematic because of the irregularity of the endomyometrial junction [40]. For the diagnosis of myometrial invasion, clear evidence of irregularly distributed tumor nests within the myometrium is needed without proximity to residual nonneoplastic glands or endometrial stroma.

Squamous differentiation occurs in about 10–25 % of endometrioid carcinomas and may present as focal morula-like structures within glandular lumens (Fig. 3.1c) or as confluent sheets [41]. Squamous differentiation may be characterized by polygonal or spindle cells resembling the squamous differentiation in the uterine cervix. Other characteristics are intercellular bridges and the formation of squamous pearls. The squamous areas often show bland or slightly polymorphic nuclei. The degree of atypia of the squamous areas usually concurs with the histopathological grade of the tumor (Fig. 3.1d) [42]. Extensive immature squamous differentiation may significantly influence the histopathological grade of a carcinoma, if it is not recognized and misinterpreted as solid non-squamous growth [43]. For the distinction, it is important to take into account also the nuclear atypia of the solid area. Ki-67 might be helpful since its labeling index is low in low-grade "metaplastic" squamous areas but high in solid non-squamous structures. Poorly differentiation endometrioid carcinomas with squamous differentiation may infiltrate as small nests of atypical squamous cells or grow in sheets of atypical spindle cells resembling a sarcomatous carcinoma [41]. Extensive keratinization is rare but may be associated with keratin granulomas at various sites including outside of the uterus [44]. A subset of endometrioid carcinomas with squamous differentiation show mucinous differentiation.

The *villoglandular variant* is mostly low grade and composed of glands and delicate papillae, covered by columnar epithelium with mild to moderate nuclear atypia (Fig. 3.2a) [45]. Stage is usually low with superficial myometrial invasion. Differential diagnosis from serous carcinoma is crucial and may be challenging. The criteria will be detailed under serous carcinoma.

The rare *secretory variant* or *variant with secretory differentiation* resembles early secretory phase endometrium with glands showing sub- and/or supranuclear vacuoles (Fig. 3.3). The secretory changes may be focal or diffuse, and they may be associated with endogenous or exogenous progestins and thus be a transient change. If it occurs in premenopausal women, the adjacent endometrium may show similar changes. The secretory variant is usually low grade and predominantly glandular but may also contain solid areas and subsequently be misinterpreted as clear cell carcinoma. In contrast to clear cell carcinoma, the secretory variant of endometrioid carcinoma lacks significant nuclear atypia and other characteristic features of clear cell carcinoma [46, 47].

The *ciliated variant* is very rare although cells with apical cilia are not unusual in a not otherwise specified endometrioid carcinoma. The tumors are usually low grade and stage. There is some evidence for an association with estrogen administration [48].

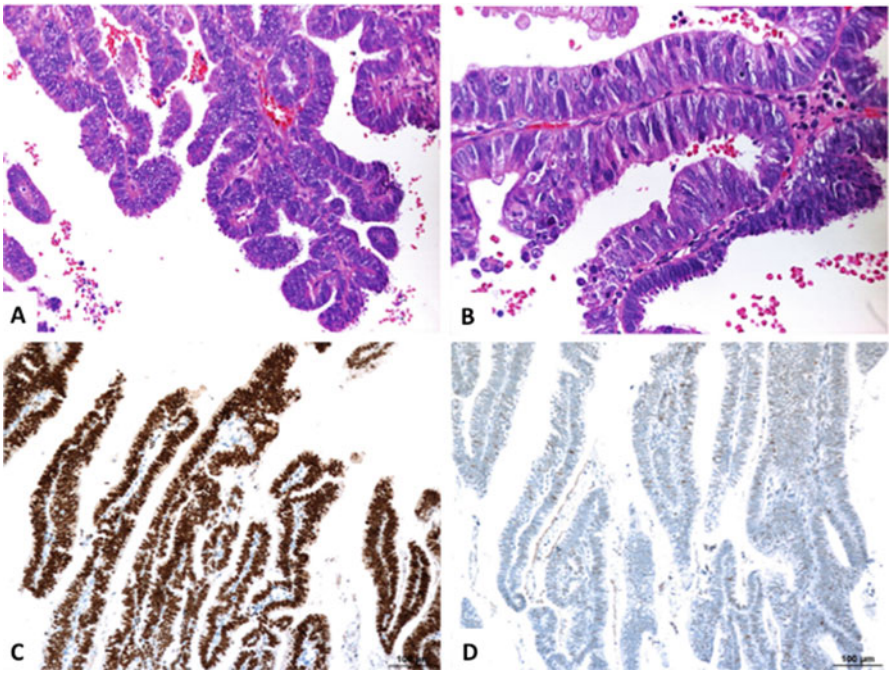

Fig. 3.2 Villoglandular variant of endometrioid carcinoma consisting of delicate papillae (**A**) covered by mildly atypical columnar epithelium (**B**). ER immunoreactivity is diffuse and strong (**C**); p53 immunoreactivity shows a wild-type pattern (**D**)

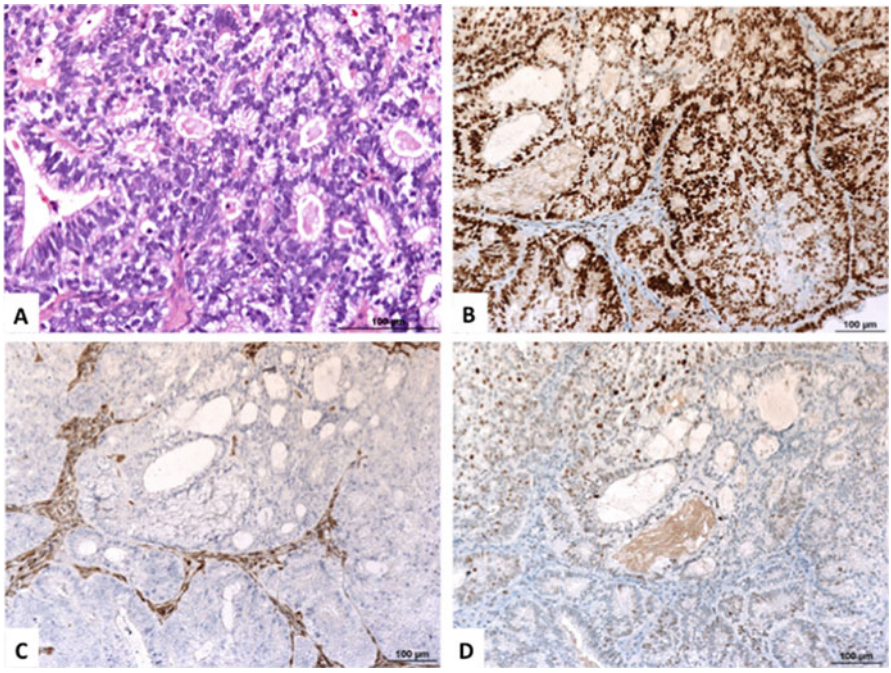

Fig. 3.3 Secretory variant of endometrioid carcinoma with glandular and solid pattern (FIGO grade 2). Note the early secretory phase cytoplasmic changes and the mild nuclear atypia (**A**). Immunoreactivity for ER is diffuse and strong (**B**), for PTEN lost (**C**), and for p53 wild type (**D**)

Differential diagnosis of endometrioid carcinoma includes atypical hyperplasia and atypical polypoid adenomyoma (APAM). The distinction from atypical hyperplasia may be particularly difficult in biopsies and curettages. The best proof of carcinoma is the evidence of invasion into the adjacent stroma or the myometrium. The presence of a confluent glandular or cribriform pattern resulting in a complex labyrinth- or maze-like appearance reflects loss of stroma and, thus, stromal invasion [49]. Other helpful criteria for invasion are a desmoplastic stromal response and extensive papillary architecture [50]. APAM consists of crowded glands often with squamous morules surrounded by a spindle cell stroma [51]. If the arrangement of the glands is complex, the differential diagnosis may be difficult, particularly since the stromal cells are of myofibroblastic origin and suggest a desmoplastic reaction. Since they usually lack desmin immunoreactivity, immunohistochemistry is not helpful for differential diagnosis between APAM and endometrioid carcinoma [52]. In contrast to endometrioid carcinoma, APAM shows an organoid pattern with a mixture of the glandular and the mesenchymal component and a lobulated appearance of the glandular component. Rarely, endometrioid carcinoma may occur in APAM and is characterized by confluent glandular growth.

Immunohistochemistry for ER and PR usually demonstrates intense positivity in low-grade (grades 1 and 2) endometrioid carcinomas but may be absent in areas of squamous differentiation. The proliferation index as measured by Ki-67 immunohistochemistry may vary. ß-catenin frequently shows aberrant (nuclear) staining and PTEN and Pax-2 staining is often reduced or lost [53, 54]. Wild-type pattern of p53 immunoreactivity showing a heterogenous mostly weak to moderate nuclear positivity with interspersed intense or negative nuclei is typical [14]. p16 immunoreactivity is heterogenous with focal intensity or it can be negative [55]. High-grade endometrioid carcinomas may show patchy intense nuclear immunoreactivity for p53 suggestive of a mutation in *TP53* [14]. ER and PR immunoreactivity may be decreased or rarely even negative; the Ki-67 labeling index is usually about 30–40 % in high-grade tumors [56, 57].

Mucinous Carcinoma

Pure mucinous carcinoma of the endometrium is rare. By definition, it needs to contain more than 50 % cells with PAS positive diastase resistant intracytoplasmic mucin [7]. More commonly, focal mucinous differentiation is found in endometrioid carcinoma, partially in combination with squamous differentiation. Cribriform or microglandular areas may rarely be present resembling microglandular hyperplasia of the uterine cervix. The histological grade and the stage at presentation are usually low. Association with exogenous estrogen has been reported [58].

Immunohistochemistry shows diffuse positivity for ER and PR and positivity for vimentin, which is helpful in the differential diagnosis to endocervical adenocarcinoma [59]. The Ki-67 labeling index is low. An important pitfall is the frequently high and diffuse immunoreactivity for p16 unrelated to HPV [60].

Serous Carcinoma

During the last three decades, serous carcinoma has been described as a distinctive disease both histologically and on the molecular level [29, 61]. The diagnostic hallmark of serous carcinoma is the combination of papillary and/or glandular architecture with high nuclear grade [7]. The histological pattern may vary by revealing both short, thick and thin, elongated papillae and glandular and solid structures (Fig. 3.4). Therefore, the term "serous papillary carcinoma" is misleading and should be avoided. The tumor cells are usually polygonal and characterized by highly atypical nuclei often with prominent nucleoli and frequent mitosis. Furthermore, the tumor cells are often irregularly arranged and form buds and tufts and are frequently detached in small groups. The surface of the papillae and the glands show prominently scalloped luminal borders. In addition, the tumor cells may also have a hobnail shape. Differential diagnosis includes villoglandular variant of endometrioid (grade 2) carcinoma and clear cell carcinoma (Table 3.3). The former shows usually thin papillae and lacks marked nuclear atypia, whereas the latter reveals at least focally cells with clear cytoplasm, hyalinized bodies, and

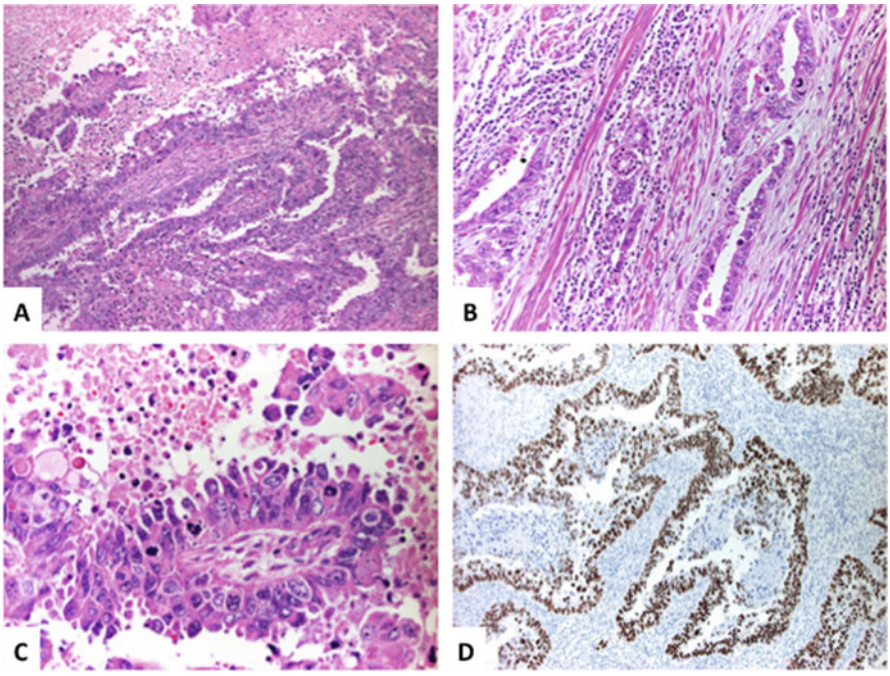

Fig. 3.4 Serous carcinoma with plump papillae (**A**) and glands (**B**) covered by markedly atypical cells (**C**) forming buds and showing loose cohesiveness. Between the papillae are areas of tumor cell necrosis (**A**). Infiltrating glands within the myometrium with inflammatory response (**B**). p53 immunoreactivity is diffuse and strong (**D**)

Table 3.3 Differential diagnosis between serous, clear cell, and endometrioid carcinoma (villoglandular variant)

	Serous carcinoma	Villoglandular variant	Clear cell carcinoma
Papillae	Variable: short, thick, densely fibrotic, or thin	Thin and delicate	Short, thick with hyaline bodies
Cells	Columnar/polygonal; proliferated with tufting and budding; detached	Columnar, pseudostratified; cohesive	Polygonal or hobnail shaped; slightly detached
Luminal borders	Scalloped	Regular ("straight")	Irregular
Nuclear features	Marked polymorphism, frequent mitosis	Mild polymorphism, infrequent mitosis	At least focally marked polymorphism, frequent mitosis
Immunohisto-chemistry	P53 diffusely positive or flat negative ER and PR negative/ focal pos. Ki-67 high	P53 wild-type/focally positive ER diffusely or heterogenously positive Ki-67 low/moderate	P53 focally positive ER and PR negative or mildly positive Ki-67 moderate to high

eosinophilic globules. Serous carcinoma occurs often in a small, atrophic uterus with atrophic endometrium and may be found within endometrial polyps. The typical patients' median age is around 65–70 years. About one half of the patients is diagnosed at higher stages (stage > I). Serous carcinoma may be associated with extensive LVSI.

Highly atypical cells may replace the surface and the glands of the adjacent atrophic endometrium, without invasion of the stroma. These changes are designated serous endometrial intraepithelial carcinoma (SEIC), the immediate precursor of serous carcinoma [62]. Under certain circumstances, particularly in biopsies, it is difficult to determine stromal invasion, and, therefore, the term minimal serous carcinoma is recommended. Biologically, SEIC is considered a noninvasive carcinoma since it may be associated with extensive extrauterine disease involving the peritoneum (e.g., omentum), the ovaries, and the fallopian tube [63]. In the setting of extensive pelvic serous carcinoma, it may be difficult to determine the site of origin. WT-1 immunohistochemistry may be helpful in the distinction between uterine and extrauterine origin since it is negative in about 90 % of uterine serous carcinomas and positive in 70–100 % of serous carcinomas from ovaries, fallopian tube, and peritoneum [64–66]. Serous carcinoma needs proper surgical staging, since stage I uterine serous carcinoma is associated with excellent outcome [67, 68].

Immunohistochemistry of uterine serous carcinoma shows a typical "all or null" immunoreactive pattern for p53 in almost all cases which strongly correlates with *TP53* mutations. The cases with a flat negative immunostaining are usually associated with frameshift mutations or a stop codon leading to truncated protein which is not detectable by the most commonly used p53 antibodies [30]. ER immunoreactivity is often weak or negative and PR immunoreactivity is often negative [56]. In cases with extensive extrauterine disease and a putative ovarian/tubal origin ER and PR immunoreactivity may be moderate to strong.

Clear Cell Carcinoma

Clear cell carcinoma is composed of polygonal or hobnail-shaped cells with clear or eosinophilic cytoplasm showing at least focally high-grade nuclear atypia [69]. The architectural pattern may be tubulo-cystic, papillary, or solid (Fig. 3.5). The papillae are short and branching with hyalinized stroma. Other typical features are densely eosinophilic extracellular globules and hyaline bodies. Like serous carcinoma, clear cell carcinoma occurs in atrophic endometrium, often within endometrial polyps [7].

Immunohistochemistry shows negative or weak positivity for ER and PR, a Ki-67 labeling index of at least 25–30 %, and frequent positivity for HNF-1β, napsin A, and racemase (AMACR) [57, 70–72]. Focal strong positivity for p53 suggestive of mutated *TP53* is found in about one third of the cases. About 30 % of the cases show loss of PTEN [26]. About 50 % of the patients are diagnosed at stages II–IV and show poor outcome with a 5-year survival of less than 50 % [69, 73, 74]. For stage I, particularly IA, an excellent prognosis was reported [75].

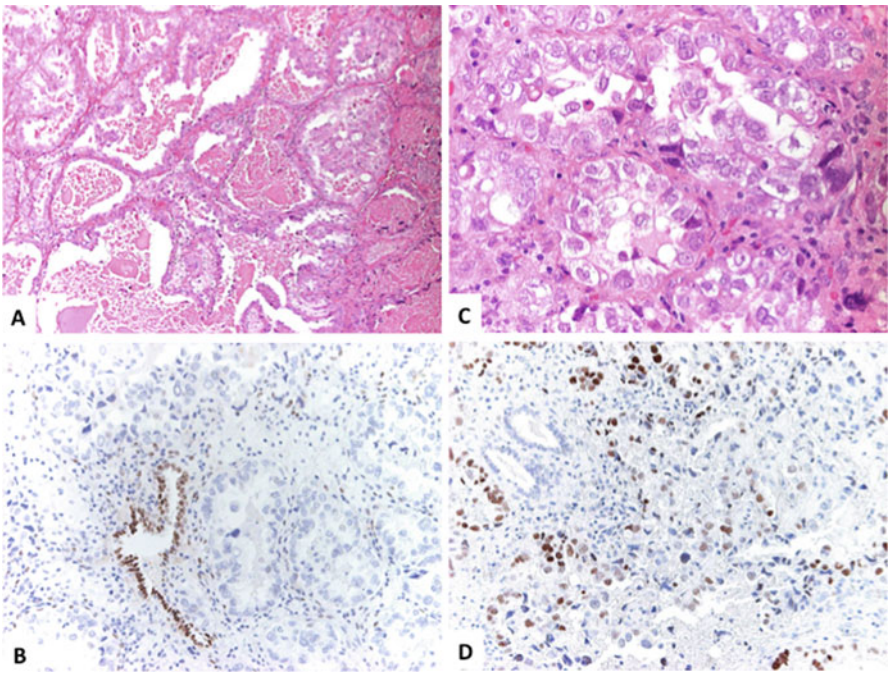

Fig. 3.5 Clear cell carcinoma with a tubulo-cystic architecture (**A**), consisting of highly atypical cells with clear cytoplasm and focal hobnail shape (**B**). Immunoreactivity for ER is negative with positivity in an entrapped atrophic gland (**C**) and shows focal strong intensity for p53 (**D**)

Mixed Carcinomas

The recent WHO consensus defined mixed carcinomas as a composition of two or more different histological types of endometrial carcinoma of which at least one is of the type II category, particularly serous and clear cell carcinomas [7]. These different tumor types should be clearly visible on H&E-stained histological sections. The minimum percentage of the minor component has been arbitrarily set at 5 %. The most frequent combinations are endometrioid and serous and endometrioid and clear cell carcinomas, respectively. Immunohistochemistry helps to support the diagnosis [76]. The high-grade component determines the prognosis even if present as minor component of 5 % [77]. It was proposed that progression from endometrioid to serous carcinoma could lead to a mixed serous and endometrioid carcinoma [12, 78].

Undifferentiated Carcinoma

Undifferentiated carcinoma is a rare epithelial neoplasm without specific morphologic differentiation. The recent WHO classification distinguishes between monomorphic undifferentiated carcinoma and dedifferentiated carcinoma [7]. The *monomorphic type* is composed of small- to intermediate-sized relatively uniform cells usually arranged in sheets. The nuclei are hyperchromatic with frequent mitosis and may show focal pleomorphism. The stroma may show a myxoid matrix resembling a carcinosarcoma (Fig. 3.6). Differential diagnosis includes other high-grade neoplasms such as high-grade sarcomas, malignant lymphoma, and neuroendocrine carcinoma [79].

The dedifferentiated carcinoma is characterized by a sharply demarcated second component, which consists of a low-grade (FIGO grade 1 or 2) endometrioid carcinoma [80]. Typically, the undifferentiated component infiltrates the myometrium, whereas the low-grade component lines the endometrial cavity. Immunoreactivity for cytokeratin may only be focally positive, whereas vimentin is diffusely positive. ER and PR are negative. Focal positivity for synaptophysin and chromogranin may be found [81].

The median patients' age is about 55 years, which may be caused by the fact that a subset of undifferentiated carcinomas occurs in patients with Lynch syndrome. The outcome is poor with greater than 50 % fatality.

Differential diagnosis includes any high-grade neoplasm of the endometrium including the biphasic carcinosarcoma (mixed malignant Mullerian tumor/MMMT). Carcinosarcomas are considered carcinomas that undergo epithelial-mesenchymal transition during their pathogenesis [82]. However, in the WHO classification, the MMMT is categorized among the mixed tumors [7]. The metastatic spread resembles carcinomas, and within metastasis MMMT may present as predominantly or purely as an epithelial neoplasm [83]. FIGO staging for endometrial carcinoma also includes MMMT. Histologically, MMMT usually contains a variety of homologous or heterologous malignant mesenchymal tissues, which are intermingled with the

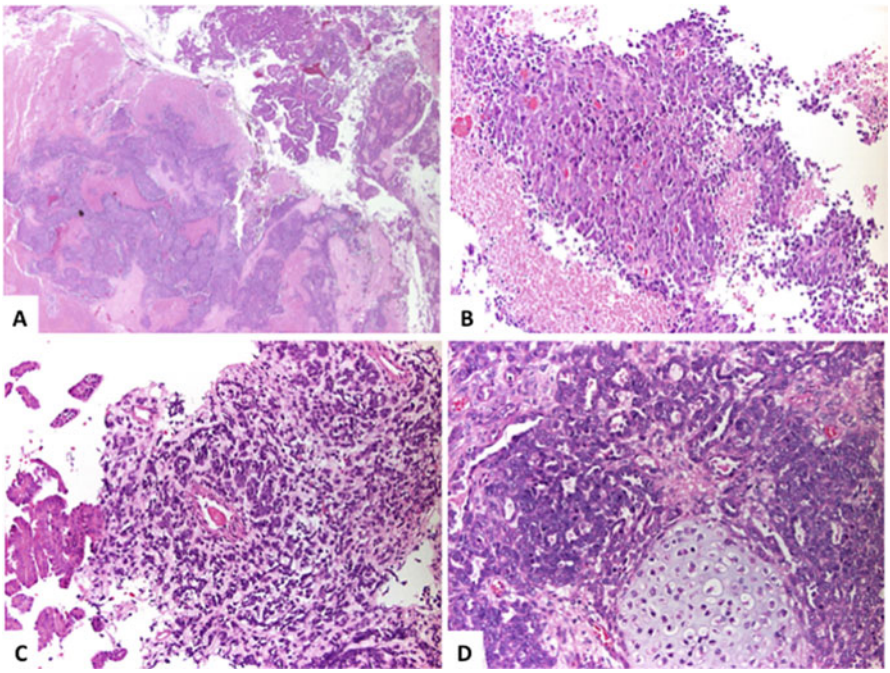

Fig. 3.6 Dedifferentiated carcinoma (**A** and **B**) and carcinosarcoma (**C** and **D**). Dedifferentiated carcinoma in curetting with a major undifferentiated and a minor well-differentiated component, which are clearly separated (**A**). The undifferentiated component resembles a small cell neuroendocrine carcinoma (**B**). Carcinosarcomas (mixed malignant Mullerian tumors/MMMTs) show a malignant mesenchymal component but may mimic dedifferentiated carcinoma (**C**). Typical is an admixture of the malignant epithelial and mesenchymal components with a variety of different patterns of differentiation (**D**)

malignant epithelial component [84]. This is in contrast to dedifferentiated carcinoma which shows a clear demarcation of the two components. In addition, the components of dedifferentiated carcinomas are less heterogenous than MMMT.

The tumor components are often but not necessarily high grade and may show a broad variety of both epithelial and mesenchymal differentiation (Fig. 3.6) [85]. The outcome is poor similar to high-grade endometrioid carcinoma and seems to be influenced by the presence of heterologous elements.

Neuroendocrine Tumors

Neuroendocrine tumors were newly defined in the recent WHO classification (Table 3.1) [81]. They are very rare and occur at a median age between 60 and 65 years. For low-grade neuroendocrine tumor (carcinoid tumor), only a few cases have been reported [86–88]. *Small cell neuroendocrine carcinoma* (*SCNEC*) resembles its counterpart from other sites (e.g., lung) [89, 90]. The growth pattern may be

diffuse nested or trabecular or show rosette-like structures. *Large cell neuroendocrine carcinoma* (*LCNEC*) consists of well-demarcated nests, trabeculae, and cords with palisading at the periphery, typically with extensive tumor cell necrosis. The tumor cells are highly atypical and show frequent mitosis. For making this diagnosis, a neuroendocrine growth pattern should be present, but may be minimal [91]. Immunohistochemistry is necessary to confirm the diagnosis with at least synaptophysin or chromogranin A positivity. CD56 (NCAM) is frequently positive but considered less specific. SCNEC shows a dot-like staining for cytokeratins. Prognosis for SCNEC and LCNEC is poor. Differential diagnosis includes other high-grade neoplasms, in particular undifferentiated carcinoma.

Grading of Endometrial Carcinoma

According to FIGO and UICC, only three grades (grades 1–3) are used for histopathological grading of gynecological cancers. For endometrioid including its variants and mucinous carcinoma, FIGO grading is used, which is based on the amount of solid non-squamous, non-morular tumor growth (Table 3.4) [92]. The presence of bizarre nuclear atypia raises the grade by one but should also raise the suspicion for serous carcinoma [93, 94]. Serous, clear cell, and undifferentiated carcinomas are by definition grade 3. Also carcinosarcomas (mixed malignant Mullerian tumors/MMMTs) are graded and categorized as FIGO grade 3. There are several problems with FIGO grading such as the recognition of small areas with solid growth, the distinction between solid squamous and non-squamous areas, and the reproducibility of bizarre nuclear atypia. Finally, the reproducibility of a three-tiered system may have its weakness. Alternative grading systems using only two tiers and partially considering patterns of growth have been proposed and subsequently validated but are not currently in use [95–98].

Staging of Endometrial Carcinoma

Endometrial carcinoma is surgically staged and, therefore, the final staging is concluded postoperatively. The current staging system as proposed by both FIGO and UICC in 2009 is detailed on Table 3.5. Several changes were made, particularly for stages I and II. Stage IA now includes carcinomas with invasion of the inner half of

Table 3.4 FIGO grading of endometrioid carcinoma of the endometrium

	Amount of solid non-squamous, non-morular growth (%)
FIGO grade 1[a]	≤5
FIGO grade 2[a]	6–50
FIGO grade 3	>50

[a]The presence of bizarre nuclear atypia raises the grade by 1

Table 3.5 FIGO/UICC classification of endometrial carcinoma

Stage		pTNM	Definition
I			Tumor confined to the uterine corpus
	IA	pT1a	No or less than half myometrial invasion
	IB	pT1b	Invasion equal or more than half of the myometrium
II		pT2	Tumor invades cervical stroma but does not extend beyond uterus
III			Local and/or regional spread of the tumor
	IIIA	pT3a	Tumor invades serosa of the uterus and/or adnexa
	IIIB	pT3b	Vaginal and/or parametrial involvement
	IIIC		Metastases to pelvic and/or para-aortic lymph nodes
	IIIC1	pN1	Positive pelvic nodes
	IIIC2	pN2	Positive para-aortic nodes with or without positive pelvic nodes
IV			Tumor invades bladder and/or bowel mucosa; distant metastases
	IVA	pT4	Tumor invasion bladder and/or bowel mucosa
	IVB	pM1	Distant metastases incl. intra-abdominal metastases and/or inguinal nodes

the myometrium, which helps in cases where assessment of myometrial invasion is difficult. Stage II is now confined to invasion of the cervical wall; tumors with only involvement of the cervical glands are classified as stage I. This revised staging system provides a simplified approach but has been challenged [99–104].

Prognostic Factors

The strongest prognostic factor for endometrial carcinoma is stage. Carcinomas confined to the uterine corpus (stage I) generally show favorable prognosis. Histological type and grade, depth of myometrial invasion, and the presence of (lymph) vascular invasion stratify this group for prognosis [105, 106]. Further adverse prognostic factors are cervical and adnexal involvement, peritoneal metastases and pelvic and para-aortic lymph node metastases [107]. Although peritoneal cytology has been excluded from staging, the presence of tumor cells in washings has been demonstrated to be an adverse prognostic factor in multivariate analysis [108]. Three different risk groups for recurrence and distant metastases have been developed by radio-oncologists for endometrial carcinomas confined to the uterus (Table 3.6) [105, 109, 110]. For more information on prognositc factors see Chap. 2.

The Clinical Approach to Endometrial Carcinoma Diagnosis

The diagnosis made on endometrial biopsy and curettage need to be as exact as possible and include type and grade to enable use of the therapeutic algorithm for endometrial carcinoma. It is crucial to recognize high grade, particularly type II

Table 3.6 Risk groups of endometrial carcinoma for recurrence and metastases

	Low risk	Intermediate risk	High risk
Stage IA	Type I, G1/G2[a]	Type I, G3	Type II
Stage IB		Type I, G1/G2	Type I, G3 Type II
Stage II		Type I, G1/G2	Type I, G3 Type II

Type I: endometrioid carcinoma including variants and mucinous carcinoma
Type II: serous, clear cell, and undifferentiated carcinoma
[a]The presence of LVSI is considered to increase the risk for recurrence from low to intermediate

carcinomas since they require extensive surgery for proper staging, provided the patient's health condition allows it. However, the accuracy of the presurgical diagnosis has limitations [111–114]. The importance of intraoperative diagnosis has decreased during the last two decades and has been replaced by intraoperative staging; particularly important is the assessment of myometrial, cervical, and/or adnexal involvement using frozen section [115, 116]. The extent of lymphadenectomy is in flux [117–121], particularly, due to the introduction of sentinel lymph node biopsy [122–124]. Therefore, intraoperative analysis of pelvic lymph nodes by frozen section with respect to determining the need for resection of para-aortic lymph nodes has lost its clinical importance [125]. The postoperative histopathological report serves as the basis for final staging.

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Part II
Molecular Profiling

Chapter 4
Traditional Approaches to Molecular Genetic Analysis

Christopher J. Walker and Paul J. Goodfellow

Abstract Molecular studies of endometrial cancer have evolved with the tools available to researchers: the methods for measuring nucleic acids, protein expression, and combinations thereof. Today "molecular genetic analysis" implies a broad range of indirect and direct tests that yield molecular phenotypes or genotypes, immunotypes, or signatures that were not conceived of when the histologic and biologic heterogeneity was first fully acknowledged.

We will provide a historical perspective on molecular genetic studies of endometrial cancers focusing on candidate genes and how early foundational research shaped both our understanding of the disease and current research directions. Examples of *direct tests* (mutation, DNA methylation, and/or protein expression) will be provided along with examples of *indirect tests* that have been and continue to be central to endometrial cancer molecular biology, such as DNA content or microsatellite instability analysis. We will highlight clinically relevant examples of molecular phenotyping and direct evaluation of candidate genes that integrate direct and indirect testing as part of routine patient care. This is not intended to be an exhaustive review but rather an overview of the progress that has been made and how early work is shaping current molecular, clinical, and biologic studies of endometrial cancer.

Keywords Indirect tests • Direct tests • Mutation testing • Candidate genes • Biologic relevance • Clinical significance

Introduction

Endometrial cancer was for many years the red-headed stepchild of oncology: unwanted and neglected. Clinically focused research has led to improved detection and treatments. Molecular biologists, however, gave little attention to endometrial cancer at the time molecular tools first became available. This is somewhat surprising

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in light of the high incidence of endometrial cancer and the remarkable increase in the number of cases associated with the use of unopposed estrogens in the 1970s. The strong link between excess estrogen and risk for development of endometrial cancer did, however, provide a solid biologic framework for correlative and descriptive molecular studies. Researchers began to formulate and test hypotheses regarding the influence of steroid hormones and their receptors on endometrial cancer biology.

Initial molecular studies of endometrial cancer were largely based on observations from other cancer types (endometrial cancer remained a "me-too" subject of investigation). The candidate gene/candidate pathway approach nonetheless yielded important insights into the pathobiology of endometrial carcinoma. Over the past two decades it has become evident that the molecular complexity of these cancers is among the highest of common tumor types studied to date. Indeed the molecular heterogeneity is consistent with the histologic and clinical variability recognized today. The rapid evolution of methods for molecular biology and informatics continues to change the perception of endometrial cancer, and its ever rising incidence has garnered the attention of epidemiologists, health care providers, and health care economists (see Chaps. 1 and 2).

DNA Content Studies

Among the earliest molecular studies of endometrial cancers were DNA content analyses that began more than 60 years ago [1]. In 1902 Theodor Boveri proposed that chromosomal defects account for cancerous phenotypes [2]. Observational studies from the 1950s and 1960s proved that the total nucleic acid content of tumor cells can differ from nonmalignant cells. Aneuploidy, referring to abnormalities in the number of chromosomes, is a "mutator phenotype" [3, 4]. It was recognized early on in the study of endometrial cancers, and the clinical diagnostic and prognostic significance of DNA content has been explored repeatedly. DNA content analysis is an indirect test that can be used to measure what is referred to as a chromosomal instability (CIN) phenotype [5]. Mauland, Wik and Salvesen [6] have recently reviewed the clinical value of DNA content assessment in endometrial cancer focusing on DNA content as a potential prognostic and predictive maker. Despite more than two decades of investigation and numerous reports on positive association between abnormalities in tumor cell DNA content and factors known to portend poor outcome, the prognostic and predictive value of DNA ploidy in endometrial cancers remains controversial [7–9]. Prospective evaluation of the prognostic and predictive value of aneuploidy is ongoing. It is conceivable that an indirect test such as DNA content measurement might be replaced by what are potentially more resolving and more powerful copy number loss or gain analyses. It is equally possible that DNA ploidy assessment combined with direct tests for mutations, epigenetic marks, changes in transcription, and altered protein expression will come to the forefront of endometrial cancer management.

DNA Mismatch Repair (MMR): Molecular Phenotyping and Direct Assessment of Candidate Genes

Endometrioid endometrial cancers have one of the highest incidences of mismatch repair (MMR) defects in human cancers studied to date. Loss of DNA MMR is associated with an easily recognized tumor phenotype, microsatellite instability (MSI). MSI is a result of somatic strand slippage mutations that have been referred to as replication errors [10, 11]. MSI analysis provides a convenient way of assessing the MMR status of tumors and falls into the category of indirect testing. When the tumor phenotype was first noted in familial colon cancers members of the conserved *mutS*, *mutH*, and *mutL* families were immediately recognized as candidate genes [12]. Loss of function alleles in *mutS*, *mutH*, and *mutL* genes in bacteria and yeast were known to lead to an accumulation of strand slippage mutations [13, 14]. In 1993, with the discovery of germline mutations in patients with familial/inherited colon cancer [15, 16], direct testing for MMR defects became possible and candidate genes were credentialed as causative factors. It was immediately obvious that carriers of MMR mutations had increased risk for endometrial cancer as well as colon cancer. This in turn spurred both direct and indirect testing for MMR defects in sporadic endometrial cancers and direct testing of candidate genes: MSI and mutation analyses. Immunohistochemistry (IHC) studies directly testing for loss of MMR proteins in tumors proceeded rapidly.

The initial studies focusing on the mutation status of candidates, specifically the *MLH1*, *MSH2*, *MSH6*, and *PMS2* genes, were disappointing. Few MSI-positive tumors had mutations [17, 18]. However, methylation of MLH1 regulatory sequences, initially seen in colon cancers with MSI, was found in the majority of MSI-positive endometrial cancers and rarely in tumors with normal MMR (no MSI or so-called microsatellite stable (MSS) tumors) [19, 20]. Aberrant *MLH1* methylation was linked to epigenetic silencing of *MLH1* based on MLH1 protein measured by IHC: tumors with methylation failed to express MLH1 [20]. *MLH1* promoter methylation thus became a direct test for a cause of MMR deficiency. Work by a number of groups confirmed methylation of *MLH1* is frequent in tumors with MSI and that germline or somatic mutations in *MLH1*, *MSH2*, and *PMS2* were seen at low frequency [17, 21, 22].

Although MMR defects associated with epigenetic silencing of *MLH1* are seen frequently in endometrial cancers, the precise mechanisms by which *MLH1* is silenced remain a matter of uncertainty. One factor contributing to *MLH1* silencing is sequence variation at or near the *MLH1* locus. Again, a candidate gene approach was pursued to test the hypothesis. In 2007, Chen and colleagues [23] provided evidence for heritable predisposition to epigenetic silencing of *MLH1*. A single nucleotide polymorphism in the 5′ untranslated regions (rs1800734) was shown to be associated with aberrant methylation of *MLH1* in both endometrial and colon cancers using a nested case study design. The finding has been confirmed in several other cohorts [24, 25]. Subsequent work in colon cancer further suggested that variation in the *MLH1* locus at rs1800734 might in fact be a low penetrance risk allele

[26]. The same association with risk has also been reported for endometrial cancer [27]. It is noteworthy that association with aberrant methylation was recently reported in peripheral blood cells [28]. This discovery has important implications for normal aging and tumorigenesis.

The study of MMR defects and endometrial tumorigenesis began as a "me too" analysis. Endometrial cancers were underappreciated or seen as a minor component of the inherited colon cancer syndromes. Today endometrial cancer is recognized as a hallmark of inherited MMR deficiency and Lynch syndrome eponym has been adopted to reflect colon, endometrial, and other tumor risk [29]. The high frequency of MMR defects (tumor MSI) in endometrial cancer spurred a range of molecular studies. One of the candidate MMR genes, *MSH6*, had been considered to play a minor role in inherited susceptibility to colon cancer. *MSH6*'s possible causative role in endometrial cancer came to prominence with a report on *MSH6* mutation in a family with Lynch syndrome in which several members were affected by endometrial cancer [30]. In 2004, a search for *MSH6* mutation in endometrioid cancers revealed frequent germline *MSH6* mutations [31]. The finding was confirmed in a second cohort shortly thereafter [32]. Today, alterations in *MSH6* are recognized as perhaps the most frequent cause of inherited endometrial cancer and clinical testing for germline *MSH6* mutation has been implemented widely for endometrial cancer patients with suspected Lynch syndrome.

Molecular testing of endometrial tumors is used in the triage for genetic testing for germline mutations. A combination of indirect and direct testing has been recommended: MSI, MMR IHC, MLH1 promoter methylation, and mutation analysis [33, 34]. Universal testing of MMR defects has been recommended by gynecologic oncology in an effort to identify patients with Lynch syndrome [35].

The link between MLH1 epigenetic silencing and endometrial tumorigenesis was firmly established in the late 1990s. The importance of loss of MMR in the initiation of endometrial cancer, be it due to inherited mutation in the context of Lynch syndrome or epigenetic silencing in sporadic endometrial cancers, was clear. In colorectal cancers, loss of MMR (MSI phenotype) was shown to be prognostic and ultimately predictive of outcome [36–38]. The discoveries in colon cancer led to similar analyses in endometrial cancer. Despite many published studies, some showing that MSI is associated with improved outcomes, others suggesting an association with reduced survival, and still other showing no effect, it is still unclear if tumor MSI is a prognostic marker. It has been suggested that both clinical heterogeneity and how MMR status is assessed and categorized (molecular lumping of indirect phenotyping of MMR status as normal or defective) may explain the differences among the different studies [39]. Bilbao-Sieyro and colleagues [40] have argued that lumping tumors into two groups, MSI-positive and MSS ignores the long appreciated variation and DNA content (ploidy) that could confound outcome studies.

The similarities and differences in MMR defects in endometrial and colon cancer have helped shape our understanding of the role MMR plays in cancer susceptibility, tumor initiation, and tumor progression. Inherited *MSH6* mutations are far more common in endometrial cancer patients than colon cancer patients. On the surface

this could be taken to mean that MSH6 is the guardian of the endometrial epithelium genome, and by extension its role in colonic epithelium less critical. However, loss of MMR due to epigenetic silencing of MLH1 is the most common cause of defective MMR in both colon and endometrial cancers and it is nearly twice as frequent in endometrial cancers than colon cancers. Clearly MMR defects help drive endometrial tumorigenesis. Molecular studies of uterine cancers focused on MMR defects will continue to rely on both direct and indirect testing methods. The Cancer Genome Atlas for uterine cancers [41] recognizes MMR deficiency as a defining feature of one of the major molecularly defined classes of endometrial cancer: tumors that have MSI and many more somatic mutations than their MMR normal counterparts. The genomic landscape of endometrial cancers is discussed in greater detail in Chap. 5.

Steroid Hormone Receptors

Aberrant steroid hormone signaling has been implicated in endometrial tumorigenesis for over a half century [42–44]. Early studies exploring the relationship between hormone receptor status and clinical parameters relied largely on radiolabeled ligand binding assays. Absence of estrogen receptor (ER) and progesterone receptor (PR) has been associated with high tumor grade, advanced stage, metastasis, and recurrence [45–48]. Today it is widely accepted that estrogen excess is associated with risk for the development of endometrial cancer [49, 50], progesterone can have antitumor activities [51, 52], and absence of the receptors on tumors appears to be associated with poor outcomes for endometrial cancer patients [53].

A major technical advance is the study of steroid hormone receptors in endometrial cancer came in 1986 when Budwit-Novotny and colleagues [54] described the use of monoclonal antibodies to detect ER and PR in tissue samples. IHC methods made it possible to distinguish between glandular and stromal expression and to determine the subcellular localization of the receptors [55, 56]. IHC analysis could also be used to conveniently study large numbers of tumors. IHC confirmed earlier reports that reduced steroid hormone receptor expression is associated with factors that portend poor outcomes in endometrial cancer patients including advanced stage, high tumor grade, advanced patient age, and presence of lymphovascular space invasion [57–61]. There are many reports on the potential prognostic significance of ER and PR expression in endometrial cancers, but to date there have been no prospective, well-controlled IHC studies [53, 62–65].

Advances in molecular biology have repeatedly changed the prism through which hormone receptors are viewed. Gene cloning and new tools for molecular biology have shown how very complex steroid hormone signaling is in normal tissues and in disease. Early IHC expression studies in endometrial cancer did not account for the multiple ER and PR protein isoforms, nor did they consider ER and PR cofactors. It is clear that estrogen, progesterone, and their receptors all play critical roles in endometrial cancer biology. In some regards it appears that the more we know, the less we understand.

There are two estrogen receptor genes, *ESR1* and *ESR2*, encoding ERα and ERβ, respectively [66, 67]. Work in many different systems has led to general acceptance that ERβ acts to oppose the actions of the canonical ERα isoform in normal tissues in breast, ovarian, and endometrial cancers [68–70]. The complexity of ERα and ERβ gene regulation makes receptor analysis in primary tissue specimens extremely challenging. Although both the alpha and beta forms bind estrogen responsive elements, they recruit different cofactors to regulate different targets or have opposite effects on the same targets [71–74]. At least three ERα and five ERβ isoforms exist and all of these are likely to play unique roles in hormone signaling [75, 76].

A single *PGR* gene exists that encodes at least seven transcripts with three established isoforms, PR-A, PR-B, and the less well-studied PR-C, along with several possible other isoforms [77–80]. Like ERα and ERβ, PR-A and PR-B have distinct molecular targets.

Candidate Tumor Suppressors and Oncogenes

TP53

The tumor suppressor gene *TP53* is the most frequently mutated gene in human cancers [81]. TP53's role in endometrial cancer has been a subject of investigation for over two decades using indirect tests (testing for allelic deletion) or direct tests for mutations or overexpression of *TP53* protein. Today it is known that TP53 is mutated in over 90% of serous endometrial cancers and is infrequently mutated in low grade endometrioid endometrial tumors [41]. However, early studies did not always make clear distinctions between type I and type II endometrial cancers or histologically different tumors as the existence of distinctive biology was not yet established.

In 1991, Okamoto and colleagues [82] first reported on *TP53* abnormalities in endometrial cancers. They tested 24 tumors for evidence of loss of heterozygosity (LOH) using Southern blot-based restriction fragment length polymorphism (RFLP) analysis with a panel of 57 markers representing all chromosomes. Five tumors had LOH on the short arm of chromosome 17 involving TP*53*. Using single strand conformation analysis and Sanger sequencing of variants, Okamoto and colleagues [82] went on to demonstrate two of these five cases with LOH also harbored *TP53* mutations as would be expected for a classical "two-hit" tumor suppressor. In the same year, it was reported that *TP53* mutations were common in endometrial cancer cell lines [83]. TP53 expression measured by IHC and indicative of *TP53* mutations was observed in 21% of endometrial cancers studied by Kohler and colleagues [84]. Collectively, the analyses in the early 1990s described earlier firmly established a role for TP53 in a subset of endometrial cancers.

The relationship between *TP53* mutation and pathologic features was further explored by Enomoto et al. [85] who assessed *TP53* mutation and LOH as well as *KRAS* mutations in endometrial cancer and atypical hyperplasia samples. *TP53* alterations were seen in ~25% of samples, including atypical hyperplasias, with a higher rate of *TP53* defects in grade 3 endometrioid endometrial cancers than in

grade 1 or 2 tumors. *TP53* and *KRAS* mutation tended to be mutually exclusive, which provided some early insights into the existence of molecularly distinct subgroups of endometrial tumors [85].

In an effort to determine if *TP53* mutations occur as early events in endometrial tumorigenesis, Kohler and colleagues investigated simple, complex, and atypical endometrial hyperplasia and carcinomas for mutations using single-strand conformational variant (SSCV) analysis coupled with direct sequencing. No mutations were identified in the hyperplasias, including 41 atypical hyperplasia specimens, and based on these findings the authors postulated that *TP53* mutation is a late event in endometrial tumorigenesis [86]. The study by Kohler and colleagues [86] did not include endometrial intraepithelial carcinoma or endometrial glandular dysplasia specimens, the putative precursors of serous endometrial carcinoma. Sherman et al. [87] reported findings for *TP53* expression (IHC status) in broad range of endometrial specimens including benign endometrium, atypical endometrial hyperplasia and endometrial intraepithelial carcinoma samples, as well as endometrioid, clear cell, and serous carcinomas. They noted positive *TP53* staining (indicative of *TP53* defects) for most endometrial intraepithelial carcinoma, clear cell, and serous samples. In contrast, only 20 % of endometrioid samples were positive, and all atypical endometrial hyperplasia and benign endometrium samples were negative. This study helped to establish that *TP53* mutation is indeed an early and frequent event in serous and clear cell endometrial carcinomas, and that mutations were less common in endometrioid tumors and rare in the histologically defined precursors of endometrioid cancer [87]. Recent studies that rely on more sensitive methods have confirmed an increasing frequency of *TP53* abnormalities with progression from normal endometrium through endometrial glandular dysplasia and endometrial intraepithelial carcinoma to serous carcinoma [88].

TP53 was one of the first candidate genes studied as a prognostic marker in endometrial cancer. Several reports suggested association between mutation status and/or positive IHC staining and features associated with poor outcome including nonendometrioid histology, advanced stage, and high grade [84, 89–91]. Subsequent studies of larger cohorts revealed TP53 status is not an independent marker of poor outcome in multivariable analyses that included histologic subtype as a confounding variable [92–95]. It is noteworthy that the rates of *TP53* mutation in endometrioid cancers reported in early studies tend to be higher than what has been reported in recent years. Possible explanations for the higher mutation rates in early studies are sample bias to larger and/or higher stage and grade tumors and misclassification of nonendometrioid tumors as *TP53*-mutated endometrioid endometrial cancers [41].

PTEN

The *PTEN* tumor suppressor is the most frequently mutated gene in endometrial cancer. Its existence and importance in endometrial cancers was first suggested by the results of deletion mapping studies (indirect tests for tumor suppressor

function). Allelic loss/deletion of the genomic region including the *PTEN* locus was recognized in endometrial cancers several years before the *PTEN* gene was cloned. In 1994, Jones and colleagues reported on loss of heterozygosity (LOH) studies in endometrial cancers with a panel of 29 microsatellite markers distributed across the genome as part of an effort to map the location of tumor suppressors. More than a third of tumors had deletion of 10q [96]. The finding of frequent 10q deletion in endometrial cancers was subsequently confirmed and the minimum region of deletion mapped to 10q23-26 [97]. In 1997 the *PTEN* gene, a novel tumor suppressor mapping to 10q23, was cloned and shown to be mutated in a range of malignancies [98, 99]. Following the initial discovery, Kong et al. examined mutation (direct testing) and LOH status of *PTEN* in a panel of endometrial, colorectal, gastric, and pancreatic carcinomas [100]. They found that mutation and LOH were seen infrequently in colorectal, gastric, and pancreatic tumors. However, among the endometrial cancers tested, 48 % showed LOH and 55 % were mutated, with most mutations resulting in clear loss of function [100]. The Kong et al. study provided the first evidence that *PTEN* is frequently mutated in endometrial cancers and strongly suggested that *PTEN* is the 10q tumor suppressor for which there is strong selection for deletion in endometrial cancers.

Around the same time, Tashiro et al. examined a panel of endometrioid endometrial cancers, serous endometrial cancer, ovarian cancer, and cervical carcinomas and found that mutation in *PTEN* is specific to endometrioid endometrial cancers [101]. A follow-up study confirmed that *PTEN* mutations are much more frequent in endometrioid than serous or clear cell endometrial cancers [102]. *PTEN* became the most commonly mutated tumor suppressor gene in endometrial cancers, and endometrial cancers garnered a great deal of attention by geneticists and cancer biologists interested in *PTEN*.

A potential link between *PTEN* mutation and MMR status was established shortly after the *PTEN* gene was discovered. MSI-positive tumors appeared to have more frequent *PTEN* mutation. Furthermore, it was initially reported that outcomes were better for women with *PTEN* mutant tumors [102]. Mutter and colleagues determined that *PTEN* defects occur early in tumorigenesis by analyzing cancers and precancers [103]. It was subsequently shown that *PTEN* lesions might precede MMR defects, which were previously established as occurring early in the development of endometrial cancers [104]. With the advent or antibodies for immunohistochemical analysis of *PTEN* expression and direct testing for defects, the Mutter lab confirmed that loss of *PTEN* protein is observed in some normal endometrial glands. They speculated that concurrent loss of *PTEN* and additional critical regulators of development may be necessary for malignant transformation [105]. Given the high frequency of both mutation and deletion of *PTEN* in endometrial cancers, it was not surprising that a search for epigenetic silencing of *PTEN* was undertaken. It has been reported that *PTEN* can also be inactivated through promoter methylation [106], but how frequently this occurs is uncertain and further methylation studies in endometrial cancers using additional methods are warranted [107].

Because *PTEN* mutation is an early event in tumorigenesis many groups have investigated the utility of *PTEN* staining in precancerous lesions to predict progres-

sion to carcinoma. Several studies suggest that there is a stepwise decrease in *PTEN* expression between normal endometrium, precancerous lesions (endometrial intraepithelial neoplasia and complex atypical hyperplasia), and endometrial cancer [103, 105, 108–111]. A large study by Lacey et al. published in 2008, on the other hand, found that *PTEN* IHC is not useful for predicting progression of atypical endometrial hyperplasia to endometrioid endometrial cancer [112]. Similar reports have found that *PTEN* negativity in endometrial intraepithelial neoplasia is not sufficient to predict malignant transformation, although combining *PTEN* status with nuclear atypia increases prediction sensitivity and specificity [113, 114]. The inconsistent findings are likely attributable to etiologic heterogeneity and the reliability of the tests used.

Traditional approaches to molecular genetic analysis include generation and characterization of genetically modified animals. The functional consequences of in vivo *PTEN* loss were first examined in 1999 by Podsypanina and colleagues who developed a knockout mouse model and observed that the *Pten*+/− heterozygous animals developed neoplasms in the endometrium, as well as liver, prostate, GI tract, thyroid, and thymus [115]. By 6 months of age, 100 % of *Pten*+/− mice exhibited endometrial hyperplasia, providing evidence to the importance of *PTEN* in this tissue [116]. Early studies combining in vivo loss of *PTEN* with other genetic alterations in cancer-associated genes determined that loss of tumor suppressors such as INK4a/ARF [117], MLH1 [118], and MIG6 [119] accelerated hyperplastic growth and led to development of carcinomas. In contrast, loss of the *Akt* oncogene in *Pten*+/− mice was found to be protective, particularly in the endometrium [120]. The *Pten* +/− mouse model was later used to show in vivo that loss of *PTEN* leads to elevated Akt activation and a subsequent increase in ER signaling that drives endometrial hyperplasia/carcinoma [121]. Interestingly, neonatal estrogen exposure was also found to be protective against endometrial hyperplasia [122]. Interest in endometrial cancer and research investments in endometrial tumorigenesis grew remarkably when *PTEN*'s role in endometrial tumorigenesis was appreciated. The endometrium became a model system in which to study perturbed signaling.

In 2008, Diakoku et al. developed an inducible uterine-specific homozygous Pten knockout using a PR (progesterone receptor) (Cre+/−) *Pten*(fl/fl) system. At the time a conditional knock out was state of the art, but today it is a traditional approach in mouse genetic analysis. Diakoku and colleagues demonstrated that homozygous deletion of *Pten* led to development of carcinomas with 100 % penetrance and early onset [123]. The model has been subsequently used to further investigate other common genetic events in endometrial cancers in vivo, in the absence of Pten. These studies have shown that endometrial carcinogenesis can be accelerated through mutational activation of Pik3ca [124], loss of Apc [125], loss of Cdh1 [126], and loss of Lkb1 [127], and that knockout of Grp78 prevents carcinoma development [128]. Today the "one gene at a time" approach for mouse models for endometrial cancer seems particularly daunting given how many genes have been implicated based on candidate gene studies alone.

The use of tumor *PTEN* protein expression to predict patient outcome and/or response to therapy has been extensively studied over the past 15 years. Complete loss of *PTEN* protein and RNA (direct tests) occurs in many patient samples, although

the reported percentage of *PTEN* negative tumors varies between 7 and 65 %, depending on the methods used and patient population investigated [9, 129–131]. The frequent involvement of a gene, such as *PTEN*, in endometrial cancer makes it an attractive candidate for therapeutics, but based on frequency alone, an unlikely prognostic marker. An early report by Mutter et al. described reduced *PTEN* protein compared to normal endometrium in most cancers investigated and 13 of 33 cases had no immunodetectable protein [103]. A similar report from Salvesen et al. found that 20 % of EC tumors examined had loss of *PTEN*, and in their study *PTEN* negativity was associated with metastasis [9]. Still another study showed that *PTEN* negative tumors tend to be less well differentiated than *PTEN*-expressing EECs [132]. The high frequency of *PTEN* abnormalities combined with the many different mutations that coexist with *PTEN* defects explains why clear pictures regarding *PTEN* status and clinical features have failed to emerge. A subgroup of *PTEN* negative tumors that also lack p27 are well differentiated and have favorable outcome [133]. Recent comprehensive mutation studies that include *PTEN* and other candidates show consistent high frequency of *PTEN* mutation or deletion in endometrioid tumors, plus or minus other common and rare mutations: these next-generation studies reflect what we began to learn by studying one candidate at a time, then combinations. Studying *PTEN* alone, as was done in early studies, gave mixed results as might be expected. *PTEN* negativity was associated with poor outcome [131, 134, 135] but there are clear contrasting reports [136, 137]. Among advanced stage patients, *PTEN* negativity is associated with favorable response to chemotherapy, and although this was first reported over a decade ago, *PTEN* status has never been used in the clinic to direct treatment strategies [138, 139]. The candidate gene *PTEN* is undeniably important in endometrial cancer. At present the prognostic and predictive significance of *PTEN* defects in endometrial cancer is entirely unknown.

KRAS

The ras family of oncogenes is frequently mutated in cancers [140, 141]. Most mutations inhibit ras GTPase activity, resulting in constitutively active ras and activation of the downstream PI3-kinase and MAP-kinase pathways. The potential role for ras family members in endometrial cancer was first investigated more than a quarter of a century ago using immunohistochemistry [142, 143]. Direct testing for the known activating mutations followed [144, 145].

Ras mutations in endometrial cancers typically are in *KRAS*, with much less frequent involvement of *NRAS* and *HRAS* [146, 147].

KRAS mutations were first identified using PCR and dot plot hybridization mutational screening for a small number of tumors, half of which harbored *KRAS* mutations [145]. Shortly thereafter *KRAS* mutation was implicated as an early event in endometrial tumorigenesis based on the observation that some endometrial hyperplasias carried *KRAS* mutations [146]. With advances in methods for mutation testing, specifically PCR amplification of tumor DNAs and allele specific

oligomer dot-blot hybridization, it was possible to analyze larger numbers of specimens and to interrogate additional base substitutions. Duggan and colleagues tested *KRAS* codons 12 and 13 for mutations in 60 endometrial cancers (a sizeable number of specimens at the time) and found that mutations were present in both the carcinomas and surrounding atypical hyperplasia [148]. The use of UV radiation fractionation to interrogate the mutation status of precancerous cells firmly established a role for *KRAS* early in endometrial tumorigenesis [148]. Additional early studies on ras mutation status in smaller numbers of cases provided a wide range of mutation frequency for *KRAS* ranging from 10 % for primary tumors to 64 % for cell lines [147, 149, 150].

There were early reports on differences in *KRAS* mutation frequency in different histologic subtypes of endometrial cancer: differences in the methods for mutation detection and histological classification of tumors likely explain some of the apparently contradictory findings for early studies. The overall consensus is that *KRAS* mutations are infrequent in nonendometrioid cancers. *KRAS* mutations, predominantly involving codon 12, are present in ~20 % of endometrioid tumors with no clear difference in mutation frequencies in tumors with intact mismatch repair and MSI-positive tumors [34, 41, 151–154].

Aberrant ras activity could provide therapeutic opportunities in endometrial cancer and although ras mutations were among the first defects described, the finding has not translated to new therapies. Pharmacologically, direct targeting of the ras family remains elusive [155], although recent efforts have shown some promise [156, 157]. The use of molecules targeting downstream ras effectors (e.g., mTOC1/2, PI3-kinase, AKT) has been explored in preclinical models and clinical trials [158]. Activation of ras in endometrial cancers may ultimately factor into treatment and even prevention strategies.

FGFR2

Members of fibroblast growth factor receptor (FGFR) family (FGFRs 1–4) play important roles in development, normal cellular processes, and pathophysiology [159]. The FGFRs are classic multifunctional receptor tyrosine kinases for which combinations of receptor isoforms and multiple ligands afford tremendous functional diversity. FGFRs activate the ras, src, and PI3-kinase pathways [160]. Kinome screens (mutation analysis of a large number of kinases) were undertaken in cancer cell lines and a variety of primary cancers with the goal of identifying druggable targets [161–163]. The FGFRs were recognized as potential oncogenes, but largely lacking cancer associations. *FGFR2*, however, became a candidate oncogene/drug target for endometrial cancers when mutations were identified in uterine cancer cell lines (http://www.sanger.ac.uk/genetics/CGP/CellLines). Mutations in *FGFR2* were first reported in primary endometrial cancers in 2007 [164]. The majority of alterations seen in endometrial cancers are missense mutations that have previously been characterized as causative germline mutations in patients with congenital

craniofacial developmental disorders (S525W and N550K as two examples) [164]. Activation of ras signaling appears to mediate the oncogenicity of *FGFR2* mutations [165, 166] and not surprisingly *KRAS* and *FGFR2* mutations are nearly mutually exclusive. The therapeutic implications for activating *FGFR2* mutations in endometrial cancer were recognized by both cancer biologists and developmental biologist [167, 168]. Efficacy of *FGFR2* inhibition was shown in endometrial cancer cell lines using the FGFR/VEGF inhibitor PD173074 as a single agent [165, 169] and in combination with doxorubicin and paclitaxel [170]. *FGFR2* thus became a viable target for therapeutic intervention in endometrial cancers: the candidate gene from a cell line screen was confirmed by simple mutation analysis in primary tumors and drug testing in cells lines. Years of work in other experimental systems, driven in large part by the importance of *FGFR2* mutations in human congenital malformation syndromes, paved the way for clinical trials in endometrial cancer using anti-FGFR agents. What, if any clinical benefit for endometrial cancer patients will come from *FGFR2* inhibitor remains to be determined. The importance of discovery of *FGFR2* activation is nonetheless important. It has further highlighted the roles of multiple signaling axes in endometrial cancers and has prompted questions regarding the function that FGFR signaling plays in the normal endometrium, precancerous endometrium, and in frank carcinoma.

Combinations of Molecular Defects Explain the Biology

Early genetic studies in endometrial cancer were performed one gene/one factor at a time. The findings from those early studies have provided both conceptual and biological frameworks for multifactor molecular approaches currently being used to characterize endometrial cancers. The idiom *nanos gigantum humeris insidentes* (discovering truth by building on previous discoveries) seems particularly apt as we begin to adopt "next-generation" technologies for molecular analysis of endometrial cancers. The increasing resolution for the cancer cell genomic landscape will have meaning only if we look back to where we have come from. Doubtless some of the giants we have already discovered (*PTEN*, MMR defects, steroid hormones, and their receptors and others) will provide important vantage points as we seek to understand the genomic complexity of individual tumors and endometrial cancers in general.

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Chapter 5
Next-Generation Sequencing

Matthieu Le Gallo, Fred Lozy, and Daphne W. Bell

Abstract Endometrial cancers are the most frequently diagnosed gynecological malignancy and were expected to be the seventh leading cause of cancer death among American women in 2015. The majority of endometrial cancers are of serous or endometrioid histology. Most human tumors, including endometrial tumors, are driven by the acquisition of pathogenic mutations in cancer genes. Thus, the identification of somatic mutations within tumor genomes is an entry point toward cancer gene discovery. However, efforts to pinpoint somatic mutations in human cancers have, until recently, relied on high-throughput sequencing of single genes or gene families using Sanger sequencing. Although this approach has been fruitful, the cost and throughput of Sanger sequencing generally prohibits systematic sequencing of the ~22,000 genes that make up the exome. The recent development of next-generation sequencing technologies changed this paradigm by providing the capability to rapidly sequence exomes, transcriptomes, and genomes at relatively low cost. Remarkably, the application of this technology to catalog the mutational landscapes of endometrial tumor exomes, transcriptomes, and genomes has revealed, for the first time, that serous and endometrioid endometrial cancers can be classified into four distinct molecular subgroups. In this chapter, we overview the characteristic genomic features of each subgroup and discuss the known and putative cancer genes that have emerged from next-generation sequencing of endometrial carcinomas.

Keywords Endometrial • Uterine • Cancer • Next-generation sequencing • Mutation • Exome • Genomic • Genetic

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Introduction

The development of sporadic and inherited forms of cancer is integrally associated with, respectively, the acquisition of somatic mutations and the inheritance of germline mutations in cancer genes, which are also referred to as "driver" genes. Since the first oncogenic point mutation was described in a human bladder cancer cell line [1–4], the search for cancer genes has often relied on identifying genes that are frequently somatically mutated across a large number of sporadic tumors, or are mutated in the germline of large cancer families and segregate with the disease phenotype. However, these types of investigations historically were hindered by the lack of a map of the human genome, and consequently a lack of understanding of the location of protein-encoding genes, and by the relatively high cost and low throughput of Sanger sequencing. Both of these bottlenecks to cancer gene discovery have now been overcome with the completion of the Human Genome Project and the development of next-generation sequencing technologies that enable rapid and affordable sequencing of exomes, genomes, and transcriptomes [5–8]. In recent years, the cancer genomics and genetics communities have embraced next-generation sequencing in their search for somatic and germline mutations that drive tumorigenesis. At the time of this writing, the efforts of many individual laboratories as well as large national and international initiatives, including The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov), the Pediatric Cancer Genome Project [9], and the International Cancer Genome Consortium [10], have mapped the mutational landscapes of at least 43 different types and subtypes of cancer, including endometrial cancer (EC) [11–16].

Endometrial cancers represent the vast majority of uterine cancers and, as such, are expected to be the seventh leading cause of cancer-related death among American women in 2015 [17]. Most endometrial cancers are carcinomas, which can be further classified into several distinct histological subtypes including serous, endometrioid, clear cell, and mixed histology tumors (See Chap. 3). The majority of deaths related to endometrial cancer are attributed to serous and endometrioid endometrial carcinomas. Historically, searches for somatically mutated genes that underlie the development of serous and endometrioid ECs used Sanger sequencing and a candidate gene approach, and involved many laboratories over the course of the past 20 years or so (see Chaps. 4 and 6). Collectively, these studies, which have been reviewed in greater detail elsewhere [18–20], delineated distinct prototypical molecular pathways that drive each subtype. *TP53* (p53) mutation or stabilization is the major driver of serous ECs [21–27], whereas mutational activation of the PI3kinase pathway is the major driver of endometrioid ECs [28–45]. In addition, *PPP2R1A* mutations [21, 46–48]; amplification or overexpression of *HER2/ERBB2* [49–59]; p16 overexpression [60–63]; decreased E-cadherin expression [64–67]; and upregulation of the genes encoding claudin 3, claudin 4, L1CAM, and EpCAM [68–70] are more common among serous than endometrioid ECs. Conversely, microsatellite instability (MSI) caused by mismatch repair defects [71], and alterations in *FGFR2* [72], the *RAS-RAF-MAPK* pathway [21, 22, 32, 73, 74], *CTNNB1*(β-catenin)

[21, 75], and *ARID1A*(BAF250A) [21, 76], are more common among endometrioid ECs than serous ECs. Despite these early successes in understanding the molecular genetic etiology of endometrial cancer, it has only been very recently, with the use of next-generation sequencing technologies, that the endometrial cancer community has been able to comprehensively document the repertoire of somatically mutated genes in serous and endometrioid ECs [11–16, 77]. A small number of endometrial carcinosarcoma exomes have recently been decoded by next-generation sequencing [78] but will not be discussed here in further detail. Rather, this chapter will review the major new insights into the mutational landscapes of serous and endometrial ECs that have been detected by next-generation sequencing.

The Genomic Landscape of Serous ECs and Copy Number High/Serous-Like ECs

Thus far, 101 serous EC exomes and their paired normal exomes have been decoded by next-generation sequencing in several independent studies [11–13, 16]. Collectively, these investigations have shown that serous ECs are characterized by widespread copy number gains and losses as well as high rates of mutations in *TP53*, *PIK3CA*, *PIK3R1*, *PTEN*, *PPP2R1A*, *FBXW7*, *CHD4*, *SPOP,* and *TAF1* signifying that these genes are likely pathogenic driver genes that contribute to the development of serous ECs [11–13, 16]. These findings confirmed long-standing observations that serous ECs tend to be nondiploid [79–85], with frequent pathogenic somatic mutations in *TP53*, *PIK3CA*, *PIK3R1*, *PTEN*, and *PPP2R1A* and extended upon this knowledge by nominating *FBXW7*, *SPOP*, *CHD4*, and *TAF1* as novel drivers of serous EC [11–13].

The integration of exome sequencing data, copy number status, and microsatellite instability (MSI) data on a large cohort (248 cases) of serous, endometrioid, and mixed histology ECs by TCGA, defined four distinct molecular subgroups referred to as ultramutated/POLE mutant, hypermutated/MSI, copy number low/microsatellite stable (MSS), and copy number high/serous-like [16]. Almost all (97.7 %) of the serous tumors in the TCGA cohort as well as 19.6 % of high-grade (G3) endometrioid ECs, 5 % of low-grade endometrioid ECs, and 75 % of mixed histology ECs were classified into the copy number high/serous-like subgroup [16]. This group is defined by high rates of copy number alteration, frequent *TP53* mutations, infrequent MSI, and a relatively low mutational burden (median of 2.3 mutations per Mb). The finding that some high-grade endometrioid ECs share molecular features with serous ECs was not surprising given the difficulty in accurately classifying some high-grade endometrial tumors as serous versus endometrioid based solely on histology [86], as well as a previous report that *TP53* mutations are significantly more common among high-grade versus low-grade endometrioid ECs ($p = 0.0046$) [21]. *TP53*, *PIK3CA*, *PIK3R1*, *PTEN*, *PPP2R1A*, *FBXW7*, and *CHD4*, all of which are known or candidate driver genes for serous ECs, were nominated as driver genes in the serous-like subgroup (Fig. 5.1) [16].

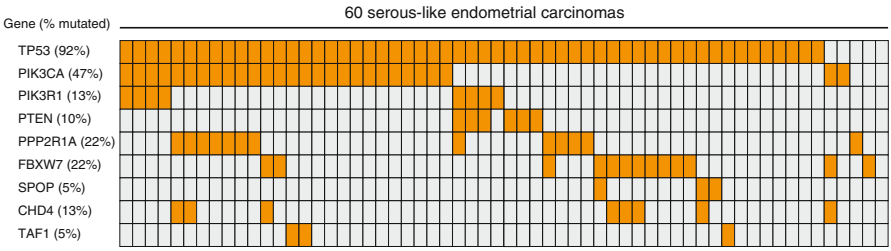

Fig. 5.1 Oncoprint displaying the occurrence of somatic mutations among nine driver genes or candidate driver genes in serous-like endometrial tumors sequenced by The Cancer Genome Atlas [16]. The mutation frequency for each gene is shown (*left*). Each vertical column represents an individual tumor. *Shaded bars* indicate the occurrence of one or more somatic mutations in a given tumor. The figure was generated from a gene query utilizing the cBIOPortal for Cancer Genomics [162]

FBXW7 and *SPOP*, two of the four novel candidate driver genes nominated among serous-like and/or serous ECs, play key roles in the ubiquitin-mediated degradation of protein substrates via the proteasome. The FBXW7 tumor suppressor protein is a critical component of the SCF^{Fbxw7} ubiquitin ligase complex, which mediates the proteasomal degradation of numerous specific protein substrates including cyclin E, c-MYC, MCL1, and NOTCH1 [87]. FBXW7 performs a dual role within the SCF complex, by binding to substrate proteins via its WD repeats and binding SKP1 via its F-box. This has the effect of bringing substrate proteins and the SKP1-CUL1-RBX1-E2 ubiquitin ligase complex into the vicinity of one another, thus facilitating the ubiquitination and subsequent proteasomal degradation of substrates, many of which are oncogenic at high levels. *FBXW7* mutations are common among a variety of human cancers and preferentially occur as missense mutations within the WD-repeat substrate-binding domain, with codons 465, 479, and 505 being prominent hotspots [88]. Cancer-associated missense mutations at these three residues can abolish binding to one or more protein substrates, in a cell-context-dependent manner [88–92]. *FBXW7* is somatically mutated in 14.7–29 % of serous ECs and 21.7 % of serous-like ECs [11–13, 93]. By comparison, it is mutated in 10–27 % of endometrioid ECs [12, 14, 16], in 7–13 % of clear cell ECs [12, 94], and in 11–25 % of mixed histology ECs [12, 16]. The occurrence of *FBXW7* mutations in concordant cases of serous endometrial intraepithelial carcinoma and serous carcinoma implicates these mutations as early genetic events in serous endometrial tumorigenesis [11]. Although the precise functional consequences of *FBXW7* mutations in the context of the endometrium remain to be elucidated, the high frequency of *FBXW7* mutations in serous and serous-like ECs, coupled with their predominant localization to the substrate-binding WD repeats (Fig. 5.2), including the three major hotspots, implies that many of these mutants probably have deleterious effects on protein function. The same argument can also be made for *FBXW7* mutations occurring in endometrioid ECs.

SPOP is a critical component of the SPOP-CUL3-RBX1 ubiquitin ligase complex, which targets a number of proteins for degradation including ERα, NCOA3/SRC3/AIB1, DAXX, AR, BRMS1, DDIT3/CHOP, and DEK [95–100]. Somatic mutations in *SPOP* occur in 7–8 % of serous ECs [12, 16], 5 % of serous-like ECs

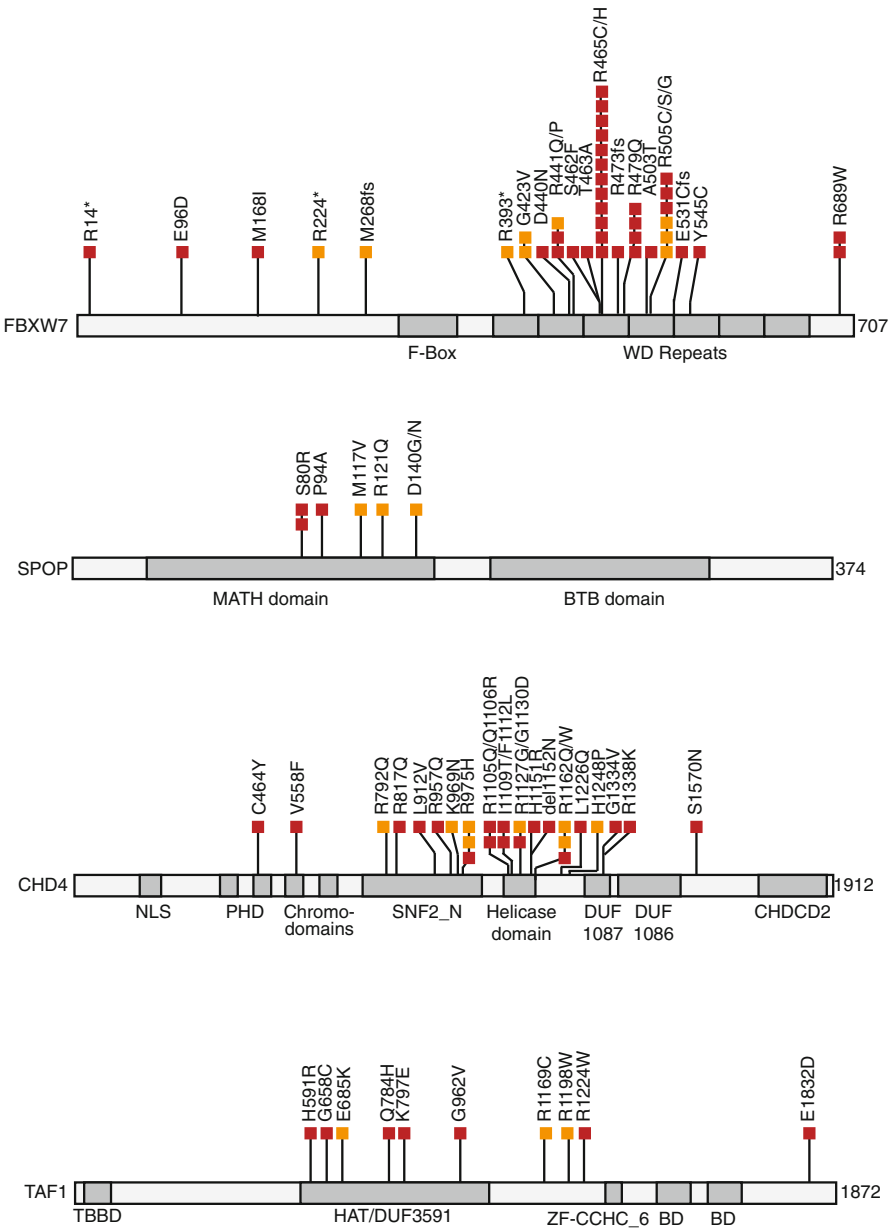

Fig. 5.2 Mutation spectra of *FBXW7*, *SPOP*, *CHD4*, and *TAF1* among serous and serous-like endometrial cancers [11–13, 16]. The position of each mutation is displayed relative to specific protein domains. Mutations in serous tumors and tumors forming the TCGA serous-like subgroup are distinguished by *dark* and *medium shading*, respectively. TCGA mutation calls were obtained from a gene query utilizing the cBIOPortal for Cancer Genomics [162]; all other mutations were manually curated from published work [11–13]

[16], 0–9 % of endometrioid ECs [12, 16], and 8 % of clear cell ECs [12]. *SPOP* mutations in serous and serous-like endometrial cancers reside in the substrate-binding MATH domain (Fig. 5.2), similar to observations in prostate cancer [101]. The predominance of somatic mutations in the substrate-binding MATH domain of SPOP in endometrial and prostate cancers is analogous to the predominance of somatic mutations in the substrate-binding WD repeats of FBXW7, suggesting that mutations in the MATH domain of *SPOP* might impair SPOP-substrate binding and lead to inappropriate accumulation of one or more protein substrates. Consistent with this idea, several SPOP MATH domain mutants uncovered in prostate cancer (Y87C/N, F102C, S119N, F125V, W131G, F133L/V) show an impaired ability to bind and facilitate the ubiquitination and eventual proteasomal degradation of AR, DDIT3/CHOP, DEK, and NCOA3/SRC3/AIB1 [95, 96, 100, 102]. However, there are notable differences in the spectrum of SPOP MATH domain mutations acquired by prostate and endometrial cancers, raising the possibility that there may also be mutation-specific functional distinctions that are cell-context-dependent. Although the functional consequences of *SPOP* mutations in endometrial cancer remain to be fully elucidated, several endometrial cancer SPOP mutations (E47K, E50K, G75R, S80R, P94A, M117I/V, and D140G) fail to regulate the ubiquitination and protein turnover of estrogen receptor alpha (ERα), leading to increased transactivation of ERα-target genes upon estrogen signaling [103].

CHD4 is a catalytic subunit of the NuRD (Nucleosome Remodeling and Deacetylation) ATP-dependent chromatin remodeling complex, which has been implicated in the regulation of gene transcription, the maintenance of genome stability, and the cellular response to DNA damage (Reviewed in [104]). The ATP-dependent helicase activity and histone deacetylase activity of the NuRD complex are provided by one of two alternative subunits, CHD3 and CHD4. Next-generation sequencing of serous EC exomes in our own laboratory led to the first description of high frequency, somatic mutations in *CHD4* among serous ECs [12], a finding that has been validated in other cohorts of serous and serous-like ECs [13, 16]. Currently, somatic mutations in *CHD4* have been documented in 13–17 % of serous ECs, 13.3 % of serous-like ECs [12, 13, 16], 7–13.5 % of endometrioid ECs [12, 16], 4 % of clear cell ECs [12], and 11–25 % of mixed-histology ECs [12, 16]. Based on high mutation rates (which consider both mutation frequency of an individual gene and the length of the coding sequence), *CHD4* has been designated a statistically significantly mutated gene (SMG) in serous ECs, serous-like ECs, and copy number low/MSS ECs, suggesting that *CHD4* mutations are likely to be pathogenic driver events in these molecular subgroups. However, the functional consequences of *CHD4* mutations in cancer are not known. Moreover, the pattern of *CHD4* mutations in serous and serous-like ECs, which consists of missense mutations dispersed throughout the protein (Fig. 5.2), makes it difficult to predict whether these are more likely gain-of-function, dominant-negative, or loss-of-function mutations. High frequency somatic mutations in other subunits of the NuRD complex have not been observed in ECs. However, copy number losses encompassing *MBD3*, a

methyl-CpG-binding domain protein within the NuRD complex, have been noted in 68 % of serous ECs [13], and deep deletions affecting MBD3 have been noted in 3 % of serous-like ECs [16].

TAF1 is a critical member of the multisubunit basal transcription factor TFIID [105]. It has been nominated as a driver gene in serous ECs [13] and is mutated in 13 % of such tumors [13]. Many *TAF1* mutations in serous and serous-like ECs localize within the histone acetyltransferase (HAT) domain (Fig. 5.2), which mediates chromatin modification and subsequent transcription. Within the TCGA cohort of ECs, *TAF1* is mutated in 14 % of tumors overall, with the majority of mutated samples being hypermutated/MSI or ultramutated/POLE mutant.

In contrast to their relatively low mutational burden, serous ECs and serous-like ECs are characterized by high-level copy number alterations [11, 13, 16]. An inverse correlation between mutation rates and copy number alterations has been observed across many different tumor types, in a pan-cancer analysis, and has given rise to the concept that tumors tend to be highly mutated (M-class tumors) or highly copy number altered (C-class tumors) but generally not both [106]. Within this analysis, most of the TCGA endometrioid endometrial tumors were classified as M-class tumors, whereas most of the serous endometrial tumors were distinguished as C-class tumors [106]. In the TCGA analysis of serous, endometrioid, and mixed histology ECs, unsupervised clustering of tumors based on copy number profiles defined four so-called copy number clusters [16]. Cluster 1 was characterized by very few copy number alterations and a high mutation rate. Cluster 2 had both broad and focal copy number alterations with peaks of amplification encompassing *CCND1* (11q13.1), and *IGFR1* (15q26.2), and deletion peaks involving the *PTEN* (10q21.31) and *WWOX* (16q23.1) tumor suppressor genes. Cluster 3 also had both broad and focal copy number alterations and was characterized by frequent amplification of chromosome 1q and associated with reduced progression-free survival ($p = 0.003$) compared to clusters 1 and 2. Cluster 4 was characterized by frequent copy number alterations; focal amplifications including *TERT*, *MECOM*, *FGFR1*, *FGFR3*, *NEDD9*, *MYST3*, *SOX17*, *MYC*, *ERBB3*, *ERBB2*, *HOXB*, *CCNE*, and *ZNF217*; and focal deletions including *RB1*, *WWOX*, *NF1*, *LRP1B*, and *PARK2* [16]. At the histological level, cluster 4 was composed of 94 % of serous tumors with the TCGA cohort as well as 24 % of high-grade endometrioid ECs, 5 % of low-grade endometrioid ECs, and 62 % of mixed histology ECs [16].

The Genomic Landscapes of Endometrioid ECs

Endometrioid ECs that are not copy number high/serous-like are distributed across the ultramutated/POLE mutant, hypermutated/MSI, and copy number low/MSS subgroups. The salient features of these three groups are reviewed in further detail in this section.

The Ultramutated/POLE Genomic Landscape

Based on whole exome sequencing, a relatively small subset of endometrial carcinomas, principally endometrioid tumors, have a so-called ultramutated phenotype [16]. The designation of these tumors as "ultramutated" reflects their overall high mutation rate (median of 232 mutations per Mb), and a characteristic mutational signature typified by an excess of T<u>C</u>T > T<u>A</u>T and T<u>C</u>G > T<u>T</u>G nucleotide substitutions [16, 107, 108].

Mechanistically, the ultramutable phenotype of sporadic ECs is attributed to defective DNA repair during replication, caused by the presence of somatic mutations in the proof-reading, exonuclease domain of *POLE*, which encodes the catalytic subunit of the Pol-ε holoenzyme that mediates lagging strand DNA synthesis during replication [16, 109]. Mutations outside the exonuclease domain of *POLE* are also present in endometrial tumors sequenced within the TCGA cohort but these mutations are not associated with an ultramutator phenotype [16]. Therefore, as a proxy for the ultramutated phenotype, follow-up mutational studies of *POLE* in EC have relied on targeted sequencing of exons 9–14, which encode the entire exonuclease domain, or exons 9 and 13, which encode residues 268–303 and 410–445 and encompass two major mutational hotspots at codon 286 and codon 411 [110–113]. To date, the *POLE* exonuclease domain has been completely or partially sequenced in ~2400 ECs, revealing a mean somatic mutation frequency of 6.5 % (range 2.7–15.1 %) among 2115 endometrioid ECs, and of 4.1 % (range 0–25 %) among 217 nonendometrioid ECs [109–113]. In some studies, *POLE* exonuclease domain mutations were significantly associated with high tumor grade [109, 110] and younger age at diagnosis [110, 113].

There has been considerable interest in evaluating whether *POLE* mutations have prognostic significance in EC following TCGA's initial observation that patients in the ultramutated/POLE subgroup have an improved progression-free survival compared with the hypermutated/MSI, copy number low/MSS, or copy number high/serous-like subgroups [16]. Thus far, the weight of evidence supports *POLE* mutations as a favorable prognostic indicator for EC, particularly for endometrioid ECs, based on studies in patient populations enriched for high-grade tumors and/or tumors from patients considered to be at intermediate–high risk of recurrence [110–112].

In the first such study, Meng et al. found somatic *POLE* (exons 9–14) mutations in 15 % of 53 high-grade (G3) endometrioid ECs. A combined analysis of this tumor series with 49 G3 endometrioid ECs in the TCGA cohort noted that *POLE* mutations were a significant ($p = 0.010$) independent prognostic indicator of improved progression-free survival in a multivariate analysis adjusting for age and stage [112]. There was also a trend toward an association between *POLE* mutations and improved disease-free survival among G3 endometrioid ECs but this did not achieve statistical significance. In terms of overall survival, *POLE* mutations in the combined set of 99 G3 endometrioid ECs were a significant indicator of improved overall survival in univariate analysis ($p = 0.046$) but the association did not reach statistical significance in a multivariate analysis ($p = 0.053$) corrected for age and stage.

The TransPORTEC consortium recently determined the *POLE* (exons 9 and 13), *TP53*, and MSI status of 118 high-risk ECs included in the PORTEC-3 clinical trial (US NCI ClinicalTrials.gov identifier: NCT00411138) and used these markers as surrogates to stratify tumors into subgroups resembling ultramutated (POLE-mutant), hypermuted (MSI+) and serous-like ECs (TP53 mutant), as well as an unclassified group referred to as NSMP (no specific molecular profile). *POLE* exon 9 and exon 13 mutations were found in 12 % of all high-risk tumors (14 of 116) and 16 % of high-risk endometrioid tumors (14 of 86). *POLE*-mutated cases and MSI+ cases had more favorable clinical outcomes than p53-mutated cases or NSMP cases. When all histological subtypes were considered, 5-year recurrence-free survival rates for *POLE*-mutated cases and MSI+ cases were significantly higher than for p53-mutated cases and NMSP cases ($p < 0.001$). When only endometrioid tumors were considered, *POLE*-mutated cases and MSI+ cases were associated with higher rates of recurrence-free survival ($p = 0.004$) and distant metastasis-free survival ($p = 0.004$) [111]. Based on these findings, it has been speculated that molecular profiling might serve as an informative adjunct, to clinicopathological features, for risk assessment and subsequent clinical management [111].

The relationship of *POLE* exons 9 and 13 mutations with clinicopathological variables has also been evaluated in a large series ($n = 788$) of EC cases within the PORTEC-1 and PORTEC-2 clinical trials [110]. PORTEC-1 recruited patients with stage I disease and intermediate risk of recurrence [114], whereas PORTEC-2 recruited patients with stage I/IIA disease and high–intermediate risk of recurrence [115]. Within the combined PORTEC-1/2 cohorts, somatic mutations in *POLE* were found in 6.1 % of endometrioid ECs (47 of 770 tumors) and in 5.5 % of nonendometrioid ECs (1 of 18 tumors) [110]. Overall, *POLE* mutations were statistically significantly associated with an earlier age at diagnosis ($p < 0.001$), a lower rate of lymphovascular space invasion ($p = 0.03$), a lower rate of deep (>50 %) myometrial invasion ($p = 0.045$), and high tumor grade (G3) ($p < 0.001$). Comparing mutated versus nonmutated cases, the rates of tumor recurrence (6.2 % versus 14.1 %) and disease-specific death (2.3 % versus 9.7 %) were lower among *POLE*-mutated cases, although the differences were not statistically significant, whereas no difference was observed for 10-year overall survival (76.2 % versus 70.4 %) [110]. Among G3 tumors in PORTEC1/2, *POLE* mutations were independently associated with improved recurrence-free survival in a multivariate analysis [HR = 0.11, 95 % CI = 0.001–0.84, $p = 0.03$] [110]. Furthermore, a meta-analysis of 1416 endometrial cancer cases, including those within the PORTEC1/2 trials and the TCGA study, confirmed *POLE* mutations as a favorable prognostic indicator [110]. In this meta-analysis, *POLE* mutations were statistically significantly associated with greater recurrence-free survival [HR = 0.33, 95 % CI = 0.12–0.91, $p = 0.03$] [110].

In contrast to the aforementioned studies, Billingsley et al. found no statistically significant association between *POLE* exonuclease domain mutations and clinical outcome among a large institutional-based series of ECs [113]. Possible reasons to account for the differences between the findings of this study and others could include differences in patient populations and/or interstudy differences in clinical management.

Thus far, the majority of reported *POLE* exonuclease domain mutations in endometrial cancer are accounted for by the P286R and V411L missense mutations. Interestingly, there are differences in the genomic landscapes of ECs harboring particular *POLE* exonuclease domain mutations. For example, the P286R and V411L mutants are associated with large mutational loads and a high proportion of G:C>T:A transversions, whereas the Q453R and A465V exonuclease domain mutants are associated with a much lower mutational load and fewer G:C>T:A transversions [109]. These differences are thought to reflect the relative proximity of each mutation to D275 and E277, the exonuclease catalytic residues, with mutations at closely oriented resides resulting in mutational loads and mutational signatures typical of ultramutated tumors [107, 109]. This idea is supported by a recent pan-cancer analysis that classified POLE-mutated tumors into two distinct groups, Group A and Group B [107]. Group A tumors are ultramutated, demonstrate context-dependent C>A mutations at a frequency of >20 %, and, based on structural predictions, have POLE exonuclease domain mutations localizing close to the exonuclease catalytic residues. In contrast, Group B tumors are MSI+ or MSS, have fewer than 20 % context-dependent C>A mutations, and exhibit POLE exonuclease domain mutations that in three-dimensional structural predictions are positioned away from the catalytic sites [107]. Notably, there are functional differences among mutations found in Group A tumors. Whereas some Group A mutants (S459F, P286R and P286H, and L424I) almost completely abolish exonuclease activity in vitro, others (L424V, F367S, and V411L) only reduce exonuclease activity [107]. Whether individual exonuclease domain mutants are associated with distinct clinicopathological features in EC awaits investigation.

Although the vast majority of *POLE* exonuclease domain-mutated ECs are microsatellite stable, some *POLE* exonuclease domain-mutated ECs exhibit concurrent MSI [16, 111, 113, 116]. Intriguingly, in tumors with concurrent *POLE* mutation and MSI, the MSI phenotype is generally *not* attributable to epigenetic silencing of *MLH1* by promoter hypermethylation [111, 113], which is the usual driver of MSI in sporadic ECs [117, 118]. It is estimated that somatic *POLE* exonuclease domain mutations may be present in as many as 25 % of MSI+ endometrioid ECs that lack epigenetic silencing of MLH1 [113]. Whether *POLE* mutations are a cause or a consequence of MMR defects in endometrial tumors with concurrent *POLE* mutations and MSI is not known. However, recent next-generation sequencing of malignant brain tumors arising in children with biallelic mismatch repair deficiency syndrome (also known as constitutional mismatch repair deficiency syndrome), which is linked to constitutional biallelic mutations in MMR genes [119–121], indicates that somatic *POLE* exonuclease domain mutations can occur subsequent to germline MMR defects and give rise to tumors with ultramutated genomes [122]. Thus far, somatic *POLE* mutations have not been observed in endometrial tumors arising in patients with Lynch Syndrome [113], which is linked to heterozygous germline mutations in MMR genes, leading to the idea that the *presence* of a somatic POLE exonuclease domain mutation in an endometrial tumor might be a clinically informative marker to exclude endometrial cancer patients from further germline testing for Lynch Syndrome [113].

In addition to *POLE*, another 189 genes have been nominated as candidate driver genes among the 17 ultramutated ECs described by TCGA. These include *POLE* itself, as well as several *bona fide* cancer genes such as *PTEN*, *APC*, *FBXW7*, *BRCA2*, *FANCB*, *PIK3R1*, *PIK3CA*, and *KRAS* [16]. However, given the ultramutated phenotype of these tumors, it is difficult to predict how many of the candidate driver genes are likely to be pathogenic.

The Hypermutated/MSI Genomic Landscape

All of the tumors comprising the hypermutated/MSI subgroup described by TCGA are endometrioid tumors, with 29 % of low-grade and 54 % of high-grade endometrioid tumors in the TCGA cohort falling within this group [16]. Hypermutated/MSI endometrial tumors are, as a group, characterized by high mutation rates and a microsatellite instability phenotype classified as MSI-high (MSI-H) [16]. Moreover, exome sequencing has revealed that the mutational burden of MSI-low tumors more closely resembles that of microsatellite stable tumors than of MSI-H ECs [116]. In keeping with previous observations in sporadic MSI+ endometrial carcinomas that predate next-generation sequencing [117, 118, 123, 124], the MSI phenotype of tumors in the hypermutated/MSI subgroup is almost always associated with epigenetic silencing of the *MLH1* gene, which would be expected to result in defective DNA mismatch repair and a bias toward the accumulation of somatic mutations resulting from strand slippage at nucleotide repeats. The occurrence of strand slippage mutations thus provides a mutational signature that can serve as a landmark to identify so-called MSI target genes.

A number of MSI target genes have been described in endometrial cancer, in studies predating the advent of next-generation sequencing [123, 125–135]. These include the mismatch repair genes *MLH3*, *MSH3*, and *MSH6*; the DNA damage response genes *DNA-PKcs*, *RAD50*, *MRE11*, *CtIP*, *ATR*, *MCPH1*, and *CHK1*, as well as *E2F4*, *BHD*, *BAX*, *IGFIIR* and *TGFβ-RII*. By necessity, these genes were selected for analysis a priori using a candidate gene approach. In contrast, next-generation sequencing of MSI+ endometrial tumors, by both TCGA and others [14, 16, 77], has provided the means to systematically search for MSI target genes, in a relatively unbiased manner [16, 77, 116]. This approach has not only confirmed an earlier finding that *ATR* is an MSI target in EC [135], but has also nominated novel MSI target genes in ECs, including *RPL22*, *CTCF, JAK1*, and *RNF43* [16, 77, 116, 136, 137], in some instances by reanalysis of the TCGA data.

RPL22 encodes a ribosomal protein and is a putative tumor suppressor gene based on observations that *Rpl22* haploinsufficiency accelerates tumorigenesis in a mouse model of T cell lymphoma [138]. In human endometrial cancer, *RPL22* is somatically mutated in 50–52 % of MSI-H endometrioid ECs, and in 37 % of the hypermutated subgroup, with >99 % of all mutations accounted for by a single frameshift mutation (c.43delA) within an $(A)_8$ mononucleotide tract (Fig. 5.3) [16, 136, 139]. *RPL22* mutations are significantly associated with a diagnosis of EC at

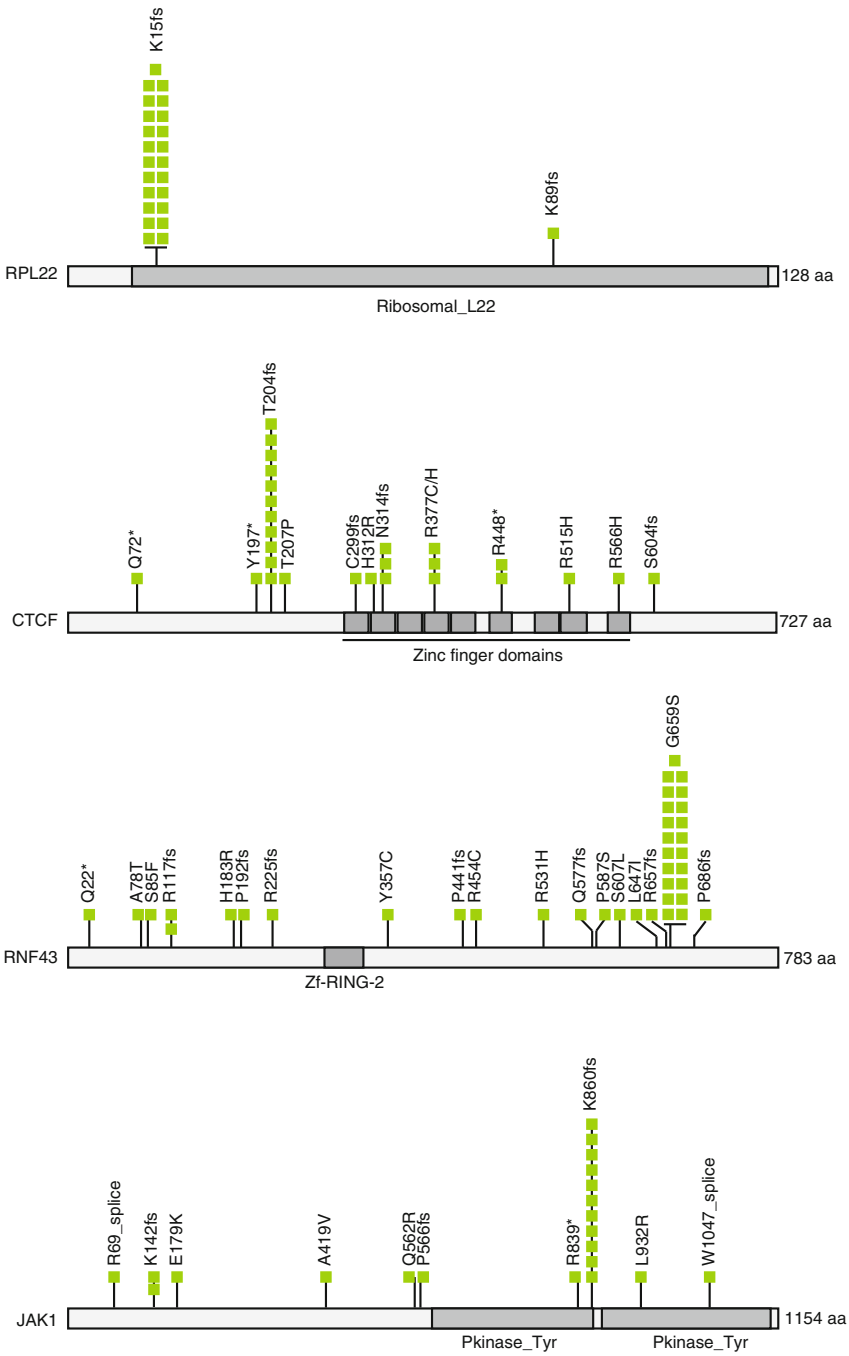

Fig. 5.3 Mutation spectra of *RPL22*, *CTCF*, *RNF43,* and *JAK1* among the hypermutated/MSI subgroup of endometrial tumors defined by The Cancer Genome Atlas (TCGA) [16]. The position of each mutation is displayed relative to specific protein domains. The mutations were identified by TCGA [16] and/or by subsequent reanalysis of TCGA data by other groups [77, 116, 137]. TCGA mutation calls were obtained from a gene query utilizing the cBIOPortal for Cancer Genomics [162]; all other mutations were manually curated from published work [77, 116, 137]

later age (67 years versus 63 years; $p=0.005$); however, no correlation has been observed between *RPL22* mutational status and clinical outcome [136]. The lack of association with outcome has led to speculation that *RPL22* mutations might either be nonpathogenic passenger mutations or, conversely, pathogenic driver mutations that are important in tumor initiation rather than tumor progression [136].

The CTCF zinc finger protein binds chromatin on numerous sites throughout the genome, mediates long-range interactions across the genome, functions as an insulator protein, and regulates chromatin architecture as well as multiple facets of transcription [140, 141]. Strand-slippage mutations in *CTCF* were first described in MSI+ endometrial cancers by Zighelboim et al., in the exomes of MSI+ endometrioid ECs associated with disease recurrence, and on subsequent reanalysis of the TCGA data [77]. Overall, *CTCF* mutations have been noted in 35 % of MSI+ endometrioid ECs compared with 25 % of MSI- endometrioid ECs. The majority of *CTCF* mutations in MSI+ cases are frameshift mutations resulting from strand slippage. The recurrent $CTCF^{T204fs}$ mutant accounts for 25 % of all *CTCF* mutations in MSI+ endometrioid ECs but has not been detected among MSS ECs [77]. Mutant *CTCF* transcripts appear to be subject to nonsense-mediated decay, suggesting that CTCF may function as a haploinsufficient tumor suppressor in MSI+ endometrial cancer [77].

The *RNF43* E3 ubiquitin ligase is a putative tumor suppressor based on its frequent mutation in pancreatic intraductal papillary mucinous neoplasms and mucinous cystic neoplasms [142], and its ability to negatively regulate WNT/β-catenin signaling by mediating the degradation of the Frizzled receptor [143]. *RNF43* mutations were first noted in endometrial cancer among 27 % of endometrioid tumors that were whole-exome sequenced by Kinde et al. [14], with a higher incidence of mutations among MSI-H endometrioid ECs than in MSS endometrioid ECs (50 % versus 14 %, respectively). All *RNF43* mutations in MSI-H endometrioid ECs, and one of two mutations in MSS endometrioid ECs, are represented by a single frameshift mutation (G659fs) [14]. These initial observations were confirmed in a reanalysis of the TCGA dataset by Giannakis et al., who noted somatic mutations of *RNF43* in 50.7 % of MSI-H ECs versus 4.6 % of MSI-L/MSS endometrioid ECs [137]. Two-thirds of these mutations are frameshift mutations, of which the vast majority (72.2 %) are represented by the G659fs mutation, which is also a mutation hotspot in MSI+ colorectal carcinomas and gastric carcinomas [137, 144]. Since RNF43 negatively regulates WNT/β-catenin signaling, it is anticipated that somatic frameshift mutations in this gene likely activate the WNT/β-catenin pathway [137], which is perturbed in 20 % of hypermutated/MSI endometrioid ECs as a result of somatic *CTNNB1* mutations [16].

The JAK1 kinase mediates JAK-STAT signal transduction in response to various cytokines including interferon-gamma [145]. *JAK1* undergoes frameshift mutations in 30 % of MSI-H endometrial tumors [116], and in 9.5 % of unselected endometrial tumors [146]. The mutations reported in EC include three that form hotspots ($JAK1^{K142fs}$, $JAK1^{P430fs}$, $JAK1^{K860fs}$) either among endometrial cancers or among ECs and other gynecological cancers [146]. The corresponding transcripts appear to be stable suggesting that these mutations encode loss-of-function JAK1 mutants that lack, at a minimum, the C-terminal kinase domain [146]. The presence of *JAK1*

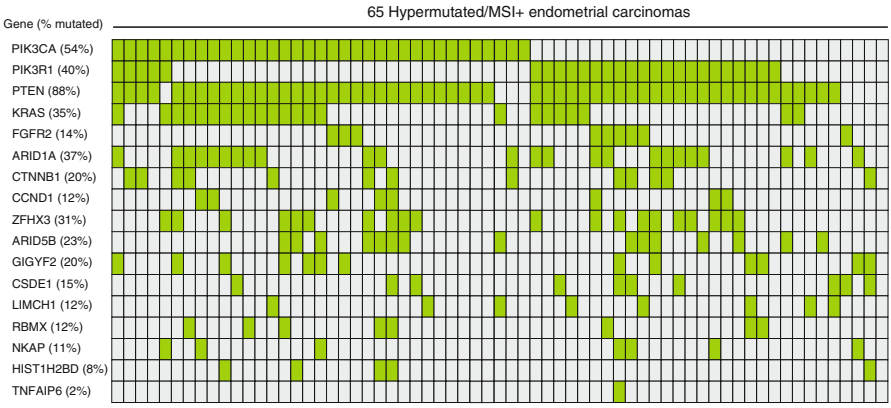

Fig. 5.4 Oncoprint displaying the occurrence of somatic mutations among 17 driver or candidate driver genes in hypermutated/MSI endometrial tumors sequenced by The Cancer Genome Atlas [16]. The mutation frequency for each gene is shown (*left*). Each vertical column represents an individual tumor. *Shaded bars* indicate the occurrence of one or more somatic mutations in a given tumor. The figure was generated from a gene query utilizing the cBIOPortal for Cancer Genomics [162]

frameshift mutations in MSI-H ECs correlates with transcriptional repression of downstream genes suggesting that these mutations functionally impact JAK-STAT signal transduction pathways [116]. Consistent with this idea, interferon-gamma stimulated expression of *LMP2* and *TAP1* is impaired in EC cell lines bearing *JAK1* frameshift mutations. Moreover, EC cell lines harboring endogenous JAK1 mutations appear to be defective in MHC class I (HLA-ABC) antigen presentation following INF-gamma stimulation [146]. Consequently, it has been suggested that the role of JAK1 frameshift mutations in EC might be to facilitate the escape of tumor cells from immune surveillance [146].

In addition to genes that are considered to be MSI targets, another 17 protein encoding genes have been nominated as driver genes in the hypermutated/MSI EC subgroup [16]. These additional genes consist of *PTEN*, *PIK3CA*, *PIK3R1*, *KRAS*, *FGFR2, ARID1A*, *CTNNB1*, and *CCND1*, which were established as drivers of endometrioid endometrial cancer in early studies that preceded next-generation sequencing [21, 22, 28, 30–34, 45, 72, 76, 147–159], as well as *ZFHX3*, *ARID5B*, *GIGYF2*, *CSDE1*, *LIMCH1*, *RBMX*, *NKAP*, *HISTIH2DB*, and *TNFAIP6*, which had no previously known role in EC (Fig. 5.4) [16].

PTEN, *PIK3CA*, and *PIK3R1* are critical regulators of the PI3kinase signal transduction pathway, which mediates cell survival, growth, metabolism, and motility (reviewed in [160]). Collectively, *PTEN*, *PIK3CA*, and *PIK3R1* undergo mutations or copy number alterations in 95.4% of hypermutated/MSI ECs. We previously found that endometrial tumors have a unique distribution of mutations, compared to other types of cancer, with as many mutations localizing to the amino terminal ABD and C2 domains as to the carboxy-terminal helical and kinase domains that encompass three strongly oncogenic hotspot mutations (E542K, E545K, and H1047R) [32]. Interestingly, these three hotspot mutations, as well as several other mutations

of strong or intermediate oncogenicity, occur significantly more often among *PIK3CA*-mutated/MSS-ECs (62.1 %) than among *PIK3CA*-mutated/MSI-ECs (29.7 %) ($p < 0.003$) [161].

KRAS and *FGFR2*, which regulate signal transduction via the RAS-RAF-MEK-ERK pathway, are mutated in 35 % and 14 % of hypermutated/MSI ECs, respectively, in a mutually exclusive manner. These observations are consistent with previous findings of frequent mutations in *KRAS* (28 %) and *FGFR2* (15–22 %) among MSI+ endometrioid ECs, which tended to be mutually exclusive one with another [72, 147]. Overall, *KRAS* or *FGFR2* alterations were more frequent among hypermutated/MSI ECs than copy number low/MSS tumors (49 % versus 28 %, respectively) [16, 162].

The *ARID1A* tumor suppressor gene encodes the BAF250A subunit of the SWI-SNF chromatin-remodeling complex and is mutated in 36.9 % of hypermutated/MSI ECs [16]. The incidence of *ARID1A* mutations is similar between the hypermutated/MSI subgroup and the copy number low/MSS subgroup (35 % versus 42 %, respectively) [16]. This is perhaps somewhat unexpected given that other studies in EC have noted a positive association between loss of BAF250A expression by immunohistochemistry and MSI+, suggesting that epigenetic modification of MLH1 leading to MSI might be a consequence of BAF250A loss [163–165].

The heteromeric ARID5B–PHF2 complex is a chromatin-remodeling complex that regulates gene transcription [166]. *ARID5B* is mutated in 23 % of hypermutated/MSI ECs (23 %) but in only 6 % of copy number low/MSS ECs [16, 162]. Many (56 %) of the *ARID5B* mutations in EC are either frameshift mutations or nonsense mutations carboxy-terminal to the ARID domain, suggesting loss of function or haploinsufficiency. Although the functional consequences of somatic *ARID5B* mutations in endometrial cancer are unknown, germline variants in *ARID5B* are associated with increased risk of childhood acute lymphoblastic leukemia [167–171], and somatic microdeletions in *ARID5B* have also been observed in this malignancy [172].

ZFHX3/ATBF1 encodes a homeotic transcription factor [173, 174] and is somatically mutated in 30.8 % of hypermutated/MSI ECs compared to 2 % of copy number low/MSS ECs. In hypermutated/MSI tumors, 60 % of mutations are frameshift mutations predicted to encode truncated proteins lacking at least one of four homeobox domains. Somatic mutations in *ZFHX3/ATBF1* are frequent in prostate cancers, in which it has been suggested to function as a tumor suppressor gene [175]. Consistent with this idea, mice with homozygous or hemizygous deletion of *Atbf1* in prostate epithelial cells develop prostatic hyperplasia and intraepithelial neoplasia [176].

GIGYF2 (PARK11) is a Grb10-interacting protein that regulates IGF1-mediated activation of ERK1/2 via IGF-IR [177–179] and regulates protein translation when complexed with m4EPH [179]. The gene is mutated in 20 % of hypermutated/MSI ECs but not in copy number low/MSS ECs [16]. There is a large body of evidence both supporting and refuting *GIGYF2* as a causative gene for familial Parkinson's disease [180, 181]. To date there is no established role for *GIGYF2* in tumorigenesis.

CSDE1/*UNR* encodes an RNA-binding protein [182] and is mutated in 15.4 % of hypermutated/MSI ECs compared to 1 % of copy number low/MSS ECs [16]. Approximately half of all *CSDE1*/*UNR* mutations in hypermutated/MSI ECs are located within the cold-shock domains, which contain RNA-binding motifs.

LIMCH1/LMO7B is mutated in 12.3 % of hypermutated/MSI ECs, but not in copy number low/MSS ECs. A single frameshift mutation (LIMCH1^{R421fs}), located amino terminal to the LIM domain, accounts for 75 % of *LIMCH1* mutations in hypermutated/MSI ECs. Little is known about the function of LIMCH1/LMO7B.

RBMX is an X-linked gene that encodes an RNA-binding protein that has been implicated in RNA splicing, transcriptional regulation, the DNA damage response, and sister chromatid cohesion [183–186]. *RBMX* mutations are present in 12.3 % of hypermutated/MSI ECs, with the majority of mutations represented by a single in-frame deletion (MVEAdelWLK). No *RBMX* mutations have been reported in copy number low/MSS ECs.

The NKAP transcriptional repressor acquires somatic mutations in 12.8 % of hypermutated/MSI ECs compared with 1 % of copy number low/MSS ECs. *HISTIH2BD* (histone cluster 1 H2BD) is mutated in 7.7 % of hypermuated/MSI ECs. Most of the mutations within *NKAP* and *HISTIH2BD* in hypermutated cases are missense mutations dispersed throughout the coding region. *TNFAIP6* mutations are rare in both hypermutated/MSI+ ECs and copy number low/MSS ECs (1.5 % and 1 %, respectively).

The Copy Number Low/Microsatellite Stable (MSS) Genomic Landscape

Almost all tumors within the copy number low/MSS EC subgroup are of the endometrioid subtype [16]. Overall, the tumors that formed this subgroup represented 60 % of low-grade endometrioid ECs, 8.7 % of high-grade endometrioid ECs, 2.3 % of serous ECs, and 25 % of mixed histology ECs within the TCGA cohort [16].

The unifying features of copy number low/MSS ECs are few somatic copy number alterations, microsatellite stability (MSS and MSI-low), frequent mutations in *PIKCA-PIK3R1-PTEN* (92 %), *ARID1A* (42 %), *CTNNB1* (β-catenin) (52 %), and *SOX17* (8 %) (Fig. 5.5). The frequency of *PIK3CA-PIK3R1-PTEN* alterations and *ARID1A* alterations is comparable between copy number low/MSS ECs and hypermutated/MSI ECs [16]. In contrast, *CTNNB1* (β-catenin) mutations are more common among copy number low/MSS tumors than hypermutated/MSI tumors (52 % versus 20 %) [16]. Similarly, *SOX17* mutations are a unique attribute of the copy number low/MSS tumors and are not present among hypermutated/MSI tumors (7.8 % versus 0 %). The increased prevalence of *CTNNB1* mutations among copy number low/MSS ECs than among hypermutated/MSI ECs is consistent with an earlier large study that documented significantly more frequent *CTNNB1* mutations among MSS endometrioid ECs than MSI+ endometrioid ECs (24 % versus 11 %, $p = 0.002$) [147].

SOX17 is a modulator of WNT/β-catenin signaling [187–190]. With the exception of one tumor, *SOX17* and *CTNNB1* mutations are mutually exclusive among the copy number low/MSS tumors, and in aggregate these genes are mutated in 59 % of

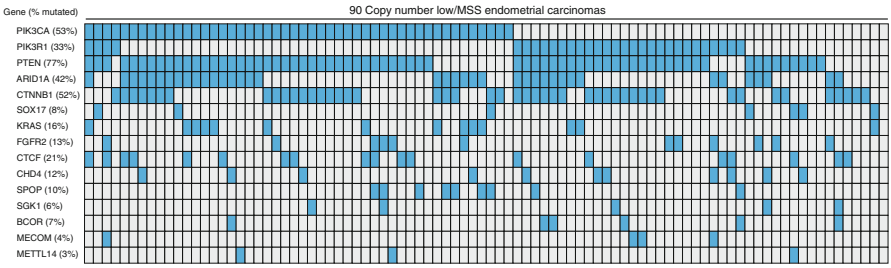

Fig. 5.5 Oncoprint displaying the occurrence of somatic mutations among 15 driver or candidate driver genes in copy number low/MSS endometrial tumors sequenced by The Cancer Genome Atlas [16]. The mutation frequency for each gene is shown (*left*). Each vertical column represents an individual tumor. *Shaded bars* indicate the occurrence of one or more somatic mutations in a given tumor. The figure was generated from a gene query utilizing the cBIOPortal for Cancer Genomics [162]

such cases. The localization of almost all of *SOX17* mutations to one of two major hotspots, one at codon 96 within the HMG box and another at codon 403 within the SOX domain, suggests these mutations are likely pathogenic gain-of-function mutants that are functionally redundant with *CTNNB1* mutations.

Unsupervised clustering of endometrial carcinomas in the TCGA tumor cohort based on gene expression data determined by RNA sequencing has discerned four major transcriptome clusters (clusters I-IV). Among these four transcriptome clusters, "Cluster II" is characterized by a high incidence of *CTNNB1* mutations (87 %), particularly within exon 3, and statistically significantly enriched expression of genes in the Wnt signaling pathway [191]. Two clusters (I and II) are enriched for low-grade and early stage cases. However, cluster II is associated with younger patients and with poorer overall survival compared to cluster I, suggesting that *CTNNB1* mutations might identify a clinically aggressive subgroup of low-grade early stage patients [191]. The association of *CTNNB1* mutation with younger patients is consistent with an early candidate gene sequencing study by Byron et al. [147].

In addition to having frequent alterations in *PIK3CA-PIK3R1-PTEN*, *ARID1A*, and *CTNNB1-SOX17*, copy number low/MSS tumors also exhibit high rates of mutation in 11 other genes, which are thus considered candidate cancer genes in this subgroup. The additional genes include *KRAS* and *FGFR2*, two well-established driver genes for endometrial cancer that tend to be mutated in a mutually exclusive manner [16, 147, 192]. Overall, the *FGFR2-KRAS* axis is mutated in 28 % of copy number low/MSS tumors compared with 49 % of hypermutated/MSI tumors. The frequency of *KRAS* mutations among copy number low/MSS tumors is lower than among hypermutated/MSI tumors (15.6 % versus 35.4 %), consistent with a previous large study of MSS and MSI+ ECs [147]. Although *FGFR2* mutations have also been observed at lower frequency among MSS tumors than MSI+ tumors (8 % versus 15 %, $p=0.016$) [147], the incidence of *FGFR2* mutations between copy number low/MSS and hypermutated/MSI subgroups is comparable (13.3 % versus 13.8 %).

The remaining candidate cancer genes in copy number low/MSS ECs consist of *CTCF*, *CHD4*, *SPOP*, *SGK1*, *BCOR*, *MECOM*, *METTL14*, and *CSMD3*. The genetic evidence supporting *CTCF*, *CHD4*, and *SPOP* as pathogenic drivers of endometrial cancer has been discussed in an earlier section of this chapter. *SGK1*, *BCOR*, *MECOM*, and *METTL14* are mutated in 6%, 7%, 4%, 3%, and 10% of copy number low/MSS ECs, respectively. *SGK1* encodes a serine-threonine kinase that is activated via PI3K-PDK1 signaling [193]. Once activated, SGK1 mediates a variety of biochemical and cellular processes that are relevant to tumorigenesis [194–208]. *BCOR* is an X-linked gene that encodes a corepressor of the BCL6 protooncogene [209]. A single missense mutant ($BCOR^{N1459S}$), located in close proximity to the ankyrin repeats, accounts for all *BCOR* mutations seen in copy number low/MSS ECs thus far [16]. Some hypermutated/MSI ECs also harbor this recurrent mutation, as well as additional mutations dispersed throughout the BCOR protein. Although somatic mutations in *BCOR* occur in other types of cancers sequenced by TCGA, the $BCOR^{N1459S}$ mutant has only been detected in endometrial tumors and lung tumors [162]. *MECOM* (MDS1 and EVI1 complex locus) encodes a zinc finger transcriptional repressor and protooncogene that is frequently activated in hematological malignancies [209]. Although only a small number of mutations have been found in *MECOM* in copy number low/MSS ECs, they all localize within zinc fingers of the protein, suggesting functional significance. *METTL14* encodes a methyltransferase that, in complex with METTL3, can methylate nuclear RNA [210]. A single recurrent missense mutation ($METTL14^{R298P}$) within the methyltransferase domain accounts for three of the four *METTL14* mutations in copy number low/MSS ECs. The recurrent nature of this mutation and its location within the functional domain of METTL14 suggest it is probably pathogenic. The nomination of *CSMD3* as a candidate driver gene in copy number low/MSS ECs, and indeed across multiple tumor types, is believed to be a false-positive finding [211].

Summary and Future Directions

The application of next-generation sequencing to decode the exomes of serous and endometrioid ECs has significantly revised our understanding of their underlying molecular etiology. The classification of these two histological subtypes into four distinct molecular subgroups has important clinical implications. As discussed, the ultramutated/*POLE*-mutant subgroup seems to define a group of high-risk patients with a relatively favorable clinical outcome and thus *POLE* mutational status might be a useful adjunct in clinical risk stratification [111]. A transcriptional subgroup, enriched with *CTNNB1* mutated tumors, identifies a group of young patients with low-grade disease and poor clinical outcome [191]. The serous-like subgroup, which shares molecular features with high-grade serous ovarian cancer and basal-like breast cancer [16], includes a relatively large fraction of high-grade endometrioid tumors [16]. This observation raises the possibility that patients with high-grade endometrioid ECs that have a serous-like molecular profile might be better managed

with chemotherapy rather than radiotherapy in the adjuvant setting [16]. Whether any of the newly described candidate cancer genes are clinically relevant remains to be seen, but the availability of a comprehensive catalog of somatic mutations for serous and endometrial cancer is hypothesis-generating and allows the scientific community to move forward with functional studies of potentially druggable genes to determine how they might be leveraged as therapeutic targets.

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Chapter 6
Endometrial Carcinoma: Specific Targeted Pathways

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Abstract Endometrial cancer (EC) is the most common gynecologic malignancy in the western world with more than 280,000 cases per year worldwide. Prognosis for EC at early stages, when primary surgical resection is the most common initial treatment, is excellent. Five-year survival rate is around 70%.

Several molecular alterations have been described in the different types of EC. They occur in genes involved in important signaling pathways. In this chapter, we

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will review the most relevant altered pathways in EC, including PI3K/AKT/mTOR, RAS–RAF–MEK–ERK, Tyrosine kinase, WNT/β-Catenin, cell cycle, and TGF-β signaling pathways. At the end of the chapter, the most significant clinical trials will be briefly discussed.

This information is important to identify specific targets for therapy.

Keywords Endometrial cancer • Signaling pathway • Target therapies • PI3K pathology

Introduction

Endometrial carcinoma (EC) is currently classified into two major groups, referred to as type I and type II, as discussed in previous chapters (Chaps. 1–4). Although this classification system is evolving in light of the recent data from next-generation sequencing of EC (see Chap. 5), the data discussed in this chapter has largely been obtained and interpreted through the lens of the current classification system.

Although the prognosis is favorable for patients with type I, early stage EC, the outcomes for patients with type II tumors (including Grade 3 endometrioid) and metastatic/recurrent tumors remain poor. After surgery (which is the most common initial treatment), the patients with tumors categorized as high risk for recurrence receive adjuvant radiotherapy and/or chemotherapy depending on the stage and type of tumor. However, traditional chemotherapeutic regimes are less effective for EC in comparison with cancers arising from other organs, emphasizing the importance of developing effective targeted therapeutic approaches for EC. However, targeted therapies have not yet been introduced in routine clinical practice.

The molecular alterations involved in the development of endometrioid carcinomas (type I) are different from those of serous carcinoma (type II). Endometrioid carcinomas show microsatellite instability, as well as mutations in *PTEN*, *KRAS*, and *CTNNB1* whereas serous carcinomas exhibit alterations of *TP53*, widespread loss of heterozygosity, as reflected by chromosomal instability as well as other

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molecular alterations. The involved signaling pathways are also different, although some of them (e.g., the PIK3 pathway) are involved in both tumor types.

In this chapter the signaling pathways most frequently affected in EC will be discussed. There will be an emphasis on the results obtained after their inhibition in in vitro assays with endometrial cancer cell lines and also in vivo assays in animal models. At the end of the chapter, the most significant clinical trials will be briefly discussed.

PI3K/AKT/mTOR Signaling Pathway

Increased PI3K/AKT/mTOR pathway activity is diagnosed in many different human cancers, as a result of overexcitation at the receptor level, loss of inhibiting PTEN function, as well as amplification or mutation in *PI3K* or *AKT* genes. Endometrioid carcinoma is the most extensively studied type of endometrial cancer, probably because of its prevalence and the availability of representative mouse models and cell lines [1–4]. Endometrioid cancers generally have high mutational load in PI3K/AKT/mTOR signaling pathway [5], probably because this pathway regulates cell growth, survival, and several cellular processes critical for cancer progression including metabolism and motility. There are three classes of PI3K enzymes grouped according to structure and function, even though class IA PI3Ks is the most clearly associated with promoting carcinogenesis [6]. Class IA PI3Ks are composed of a regulatory subunit and a catalytic subunit. Three mammalian genes *PI3KR1, PI3KR2*, and *PI3KR3* encode for the P85 and P55 regulatory subunits. Whereas the catalytic subunits isoforms (P110α, P110β, P110ϒ, or P110δ) are products of three genes *PIK3CA, PIK3CB*, and *PIK3CD*. As will be discussed in detail later, some of these genes are frequently mutated in endometrial carcinomas. Class IA PI3Ks are activated by growth factor stimulation through receptor tyrosine kinases (RTK) and alternatively by G-protein coupled receptors. This results in the transfer of phosphate groups to the inositol ring of phosphatidylinositol 4,5 bi-phosphate (PIP2) to produce the signaling molecule phosphatidylinositol 3,4,5 tri-phosphate (PIP3). This process is negatively regulated by PTEN (phosphatase and tensin homolog), which dephosphorylates PIP3 to PIP2. PIP3 propagates intracellular signaling by directly binding the pleckstrin homology (PH) domain of various signaling proteins [7]. PIP3 brings two PH domain-containing serine/threonine kinases, phosphoinositide-dependent kinase 1 (PDK1) and AKT, into close proximity. Then, PDK1 activates AKT by phosphorylation at residue Thr308 [8]. Phosphorylated AKT promotes cell survival inhibiting proapoptotic Bcl-2 family members such as BAD and BAX [9]. Phosphorylation of MDM2 by AKT antagonizes TP53 mediated apoptosis, and AKT negatively regulates forkhead transcription factors, thereby reducing production of cell death-promoting proteins. In addition, AKT also impedes negative regulation of NF-KB leading to increased transcription of prosurvival and antiapoptotic genes. AKT phosphorylates TSC2, thereby inhibiting the rheb GTPase activity of the TSC1/TSC2 dimer. Activated RHEB stimulates the

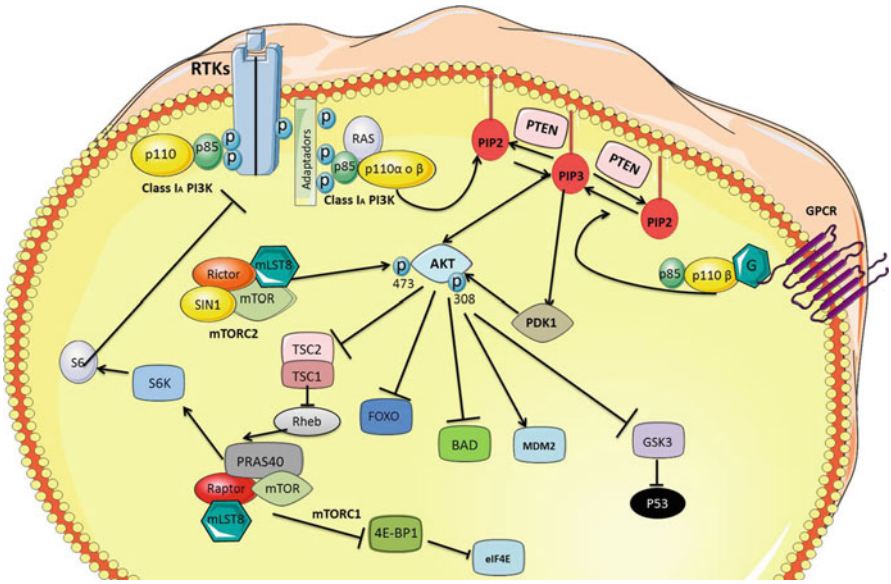

Fig. 6.1 Schematic representation of the PI3K/AKT/mTOR signaling pathway

mammalian target of rapamycin (mTOR)-containing protein complex mTORC1 leading to increase in P70s6 kinase activity. Activation of mTORC1 results in increased protein synthesis by phosphorylation of eukaryotic initiation factor 4E and the ribosomal S6 protein. At the same time that mTORC1 relays signals following PI3K/AKT activation, a second mTOR complex (mTORC2) contributes to total AKT activation by phosphorylating AKT at Ser473 [10] (Fig. 6.1).

Of note, activation of mTOR negatively feeds back to diminish PI3K activation [6]. Another mechanism of inhibiting AKT phosphorylation is through the action of the phosphatases PP2A and PHLPP [11].

Alterations in PI3K/AKT/mTOR Pathway in Endometrial Cancer

PTEN Inactivation

The *PTEN* (phosphatase and tensin homolog) gene is located at chromosome 10q23.31. The PTEN protein has a crucial role as a negative regulator of the PI3K/AKT/mTOR pathway through dephosphorylation of PIP3 at the cell membrane. Absence of functional PTEN protein leads to unopposed action of PI3K with resultant uncontrolled PIP3 production. Thus, loss or altered PTEN expression results in aberrant cell growth and apoptotic escape. *PTEN* mutations occur in a wide range of sporadic tumor types, but at high frequencies in specific tumors, including EC [12, 13]. PTEN may be

inactivated by several mechanisms. PTEN function can be compromised by genetic mutations, which result in either a heterozygous loss (50%) or a homozygous loss (100%). In addition, mechanisms including epigenetic silencing, transcriptional repression, microRNA (miRNA) regulation, disruption of competitive endogenous RNA (ceRNA) networks, posttranslational modifications, and the aberrant localization of PTEN protein can cause subtle or dramatic losses of PTEN function.

Germline and somatic mutations in *PTEN* occur mostly in the phosphatase domain, between residues 122 and 132, in exon 5 [14]. *PTEN* may also be inactivated by deletion, as shown by the elevated frequency of loss of heterozygosity (LOH). An additional proposed mechanism for *PTEN* inactivation is promoter hypermethylation. However, the true significance of *PTEN* promoter methylation has been questioned due to the possible interference of a processed *PTEN* pseudogene (*PTENP1*) with *PTEN* [15].

PTEN is mutated and lost in up to 80% of endometrioid tumors [15–18]. *PTEN* mutations have also been detected in about 55% of patients with atypical endometrial hyperplasia [19] and a subset of heterozygous *Pten* mice develop endometrioid tumors [1, 4]. Only a small percentage of type II endometrial cancers (up to 10%) show abnormalities in this gene [20].

PTEN inactivation has been proposed as an early event in the pathogenesis of EC. Generally, *PTEN* alterations occur diffusely throughout the neoplasm; however, in some other tumors, *PTEN* alterations are restricted to one or several tumor subclones. As previously stated, *PTEN* is usually regarded as an early event in EC; however, occasionally, *PTEN* alterations are also present during tumor progression, and consequently heterogeneously present in the tumor. An example of heterogeneous presence of *PTEN* alterations is EC with microsatellite instability, which represents a good scenario to assess molecular features associated with tumor heterogeneity [21].

Although somatic point mutations are the most common, germline mutations are also described; these are present in Cowden syndrome and result in a 10% lifetime risk of endometrial cancer [20].

PI3KCA Mutations

The *PIK3CA* gene, located on chromosome 3q26.3, encodes the catalytic p110α subunit of PI3K, which generates PIP3 from PIP2. Thus, alterations in *PI3KCA* gene, which is a transforming oncogene, result in increased activation of the PI3K/AKT/mTOR pathway. Moreover, mutant P110α proteins have been shown to display enhanced lipid kinase activity in comparison with the wild-type protein [5]. Activating *PIK3CA* mutations are present in about 15% of human carcinomas on average, but some differences in their incidence occur, depending on tumor type. The gain-of-function *PI3KCA* mutations present in EC depend on the tumor type. Mutations of *PIK3CA* occur in 10–30% [5, 19, 22] of endometrioid EC whereas mutations and amplifications are seen, respectively, in 35 and 46% of serous EC.

In contrast to breast and colorectal carcinomas, in which most *PI3KCA* mutations occur in two hotspots in the helicase and kinase domains [23], mutations in endometrial cancer are distributed throughout the gene [24, 25].

The presence of *PIK3CA* mutations could suggest a mechanism for carcinogenesis of EC, as an alternative to *PTEN* mutation, because both lead to an increase of PIP3 and excessive AKT activation. However, most studies of endometrial carcinoma have demonstrated frequent coexistence of *PIK3CA* and *PTEN* mutations [5], suggesting a synergic effect of both genes on AKT activation during development of endometrial tumors. It has been demonstrated that *PIK3CA* mutations occur more frequently in combination with defects in other genes functioning in the same signaling pathway such as *PTEN* or *KRAS*, which may enhance AKT activation, contributing to tumor progression [26].

Additional PI3K/AKT/mTOR Pathway Mutated Genes

PIK3R1 and *PIK3R2* genes encode the regulatory subunits P85α and P85β of PI3K and are localized in 5q12-q13 and 19p13.11 chromosomes, respectively. It has been demonstrated that *PIK3R1* mutations occur at a higher rate in EC than in any other tumor type, and *PIK3R2* is also frequently mutated [27, 28]. Gain-of-function mutations of *PIK3R2* occur in 5 % of EC whereas *PIK3R1* is somatically mutated in 20–43 % of Type I and 12 % in Type II [28]. Mutations in *PIK3R1* are preferentially localized to the P85α-iSH2 domain, which mediates binding to P110α. The high frequency and nonrandom distribution of these mutations strongly suggest that mutations of *PIK3R1* may be examples of "driver" mutations that confer a selective advantage in endometrial neoplasia.

Several PIK3R1 mutations promote an increase in AKT phosphorylation at residue Ser473, thus activating the downstream signaling pathway. It has been suggested that *PIK3R1* gain-of-function mutations could destabilize PTEN through disruption of P85α homodimerization, in support of the importance of PTEN and P85 interactions in endometrial cancer. Therefore, some authors have hypothesized that the truncating mutants of P85α are not functionally equivalent to P110α mutants [28].

AKT1 gene mutations have been described in EC at a frequency of 2.2 % in endometrioid adenocarcinomas with positive estrogen receptor and progesterone receptor expression, suggesting that these tumors are estrogen dependent. However, these tumors did not demonstrate mutations in either *PIK3CA* or *PTEN* leading the authors to suggest that *AKT1* mutations might be mutually exclusive with other PI3K-AKT activating alterations [29].

Co-mutations in different components of the PI3K pathway may also cooperate for efficient cellular transformation. PTEN protein loss and *PIK3CA* mutations have markedly different functional effects on PI3K pathway activation in some human cancers [30]. Co-mutations in PI3K pathway members occur at frequencies significantly higher than predicted in EC. For example, *PIK3CA* mutations frequently coexist with *PTEN* mutations [26].

However, *PIK3CA, PIK3R1*, or *PIK3R2* mutations are more common in cells where PTEN protein is retained, and these mutations phenocopy the functional effects of PTEN loss on downstream signaling. Mismatch repair DNA (MMR) deficiency, which is an early event in the pathogenesis of EC [31], might contribute

to these co-mutations. However, the types of mutations present in the PI3K pathway members are not characteristic of aberrations induced by MMR deficiency.

Although high AKT activity is well documented in endometrial carcinomas, very few data exist on the role of the mTOR pathway in this type of cancer; however, in vivo data show that mTOR cascade components are lacking in EC [32]. As explained before, mTOR is the catalytic subunit of two biochemical distinct molecular complexes, mTORC1 and mTORC2. Activation of mTORC1 increases translation rates and protein synthesis, affecting cell proliferation and cell survival. In this regard, Lu et al. demonstrated that dysregulation of mTOR in primary endometrial carcinomas may be achieved by loss of TSC2 and LKB1 expression (13 % and 21 %, respectively) [33].

PI3K/AKT/mTOR Signaling Inhibitors in Preclinical Studies

Our knowledge of the molecular pathways involved in endometrial neoplastic transformation supported development of novel therapeutic agents that target PI3K/AKT/mTOR pathway. Because of the prominent role of this pathway, inhibitors of PI3K/AKT/mTOR signaling have been shown to be ideal targets for anticancer therapy in vitro and in vivo in preclinical models (Table 6.1) and, in some cases, have shown promising results in clinical trials. The inhibitors of the PI3K/AKT/mTOR pathway fall into four main categories: PI3K inhibitors, mTOR inhibitors, dual mTOR/PI3K inhibitors, and AKT inhibitors.

PI3K Inhibitors

PI3K inhibitors are divided into two classes, pan-PI3K inhibitors, which inhibit all four Class I PI3Ks, or isoform-selective PI3K inhibitors. Pan-PI3K inhibitors Wortmannin and LY294002 represent the first-generation inhibitors with highly potent PI3K-inhibitory property. However, these compounds demonstrated considerable toxicities in animal studies and were not advanced to clinical trials [57]. In preclinical studies, the pan-PI3K inhibitors NVP-BKM120 and GDC-0941 have shown a reduction of cell growth in a variety of cell lines [58]. Moreover, NVP-BKM120 has demonstrated particular activity against cells with *PIK3CA* mutations [59]. In addition, GDC-0941 halted tumor progression in xenograft mice harboring a tumor developed from a *FGFR2*-mutant endometrial cancer cell line [60].

However, pan-PI3K inhibitors are blunt tools that are not specifically aligned with the disease biology and context. The main concern with pan-PI3K inhibitors is that a complete block of all class I PI3Ks for extended periods might not be tolerated. For example, NVP-BKM120 at concentrations needed to fully inhibit PI3K has off-target effects on tubulin and causes general cellular toxicity [61].

Table 6.1 Preclinical studies using PI3K/AKT/mTOR inhibitors currently under clinical trials

Compound	Experimental approach	Effect	Ref
Wortmannin: PI3K Inhibitiors (pan-inhibitors)	Analysis of Wortmannin effects on enhanced invasive phenotype of human stromal cells	Impairment of migration induced by estrogens stimulation on human stromal cells	Gentilini et al. [34]
	EGF effects in PTEN-reconstituted Ishikawa cells and correlation with Wortmannin activity	Wortmannin suppress EGF mediated cell growth in PTEN-reconstituted ishikawa cells	Tang et al. [35]
	Effects of Wortmannin in 2 EC cell lines treated with tamoxifen	Wortmannin displayed inhibitory proliferation effects in tamoxifen treated cell lines	Vivacqua et al. [36]
LY 2942002: PI3K Inhibitiors (pan-inhibitors)	In vitro/ in vivo effects of LY2942002 in Ishikawa xenograft	LY2942002 displayed cell apoptotic effects in vitro and blocked tumor growth in vivo	Guo et al. [37]
	Analysis of growth effects in 3D primary cultures of endometrial cells	Growth inhibitory effects of 3D endometrial epithelial glands	Eritja et al. [38]
NVP-BKM120: PI3K Inhibitiors (pan-inhibitors)	Analysis of NVP BKM-120 effects as a single agent and in combination with standard cytotoxic chemotherapy in a human primary endometrial xenograft model	NVP BKM-120 precludes tumor growth in a primary xenograft model. While a pattern of resistance emerges, appears to be mitigated by the addition of conventional cytotoxic chemotherapy	Bradford et al. [39]
GDC-0941: PI3K Inhibitiors (pan-inhibitors)	Analysis of GDC-0941 effects in Twenty-four human EEC	EEC cell lines harbouring *PIK3CA* and *PTEN* mutations were selectively sensitive to GDC-0941	Weigelt et al. [40]
	Analysis of GDC-0941 effects in a Pten/Lkb-1 deficient mouse model	GDC-0941 used as a single agent reduced the growth rate of primary tumor implants in Pten/Lkb1-deficient mice	Cheng et al. [41]
GSK2636771, AZD6482 and A66: PI3K Inhibitiors (PI3K P110 isoforms)	Analysis of PI3K p110 isoforms inhibitors effects in 24 human EEC	*PTEN*-mutant EEC cell lines were resistant to the p110β inhibitors GSK2636771 and AZD6482, and only in combination with the p110α selective inhibitor A66, a decrease in cell viability was observed.	Weigelt and Bissell [42]

Everolimus: mTOR inhibitors	Analysis of Everolimus effects in hyperplasia and cancer progression in BALB/C mice treated with estradiol and tamoxifen	Everolimus prevent tamoxifen-associated and estrogen-related endometrial hyperplasias in mice	Erdemoglu et al. [43]
	Effects of Everolimus in Pten heterozygote murine model	Everolimus decreases endometrial hyperplasia progression in the Pten heterozygote mice through decreased cell proliferation and increased apoptosis.	Milam et al. [44]
	Effects of Everolimus in an inducible Pten knockout mouse model	Everolimus decreases endometrial hyperplasia progression	Mirantes et al. [4]
	Effects of Everolimus in combination with Letrozole in Ishikawa cells	Everolimus inhibited cell proliferation alone, and showed synergic antiproliferative and apoptotic effects when combined Letrozole	Lu et al. [45]
	Analysis of Everolimus effects in combination with carboplatin in in vivo using AN3CA cells xenograft	Combination of Everolimus with carboplatin decreases tumor growth and protein synthesis	Korets et al. [46]
	Effects of Everolimus in combination with Gefitinib. Proliferation/apoptosis assay in Ishikawa and HEC-1A cells under estrogen-reduced conditions	Everolimus inhibited cell proliferation alone, and showed synergic anti-proliferative effects with Gefitinib	Block et al. [47]
Temsirolimus: mTOR inhibitors	Proliferation/apoptosis assay of Temsirolimus effects in NCI60 endometrial cell panel	Temsirolimus showed higher susceptibility scores in high-grade EC cell lines compared to cisplatin, doxorubicin and paclitaxel	Kharma et al. [48]
Ridaforolimus: mTOR inhibitors	Analysis of Ridaforolimus effects in combination with ponatinib in in vitro and in vivo assays using cells xenograft models	Combination of ridaforolimus and ponatinib have a synergistic effect on the in vitro growth of endometrial lines and in tumor regression in endometrial xenograft.	Gozgit et al. [49]
	Analysis of Ridaforolimus effects in vitro proliferation assay on 6 EC cell lines and in vivo using AN3CA cells xenograft	In vitro and in vivo Rifaforolimus has growth inhibitory effects in Pten-deficient cells.	Squillace et al. [50]

(continued)

Table 6.1 (continued)

Compound	Experimental approach	Effect	Ref
AZD8055: mTOR inhibitors	Analysis of AZD8055 effects in vivo using MES-SA cells xenograft	AZD8055 significant inhibited tumor growth and/or regression in uterine xenograft models	Chresta et al. [51]
	Proliferation/apoptosis assay of AZD8055 effects in 22 primary uterine serous carcinoma (USC) cell lines.	AZD8055 impairs tumor growth in c-erbB2 gene amplification USC cell lines	English et al. [52]
BEZ235: Dual mTOR/PI3K inhibitors	Analysis of BEZ235 inhibitor in comparison to Everolimus in 13 EC cell lines and in vivo using AN3CA A and HEC-59 cells xenograft	In vitro and in vivo results show an increased tumor growth suppression by BEZ235 than by Everolimus	Shoji et al. [53]
	Analysis of BEZ235 effects in a Pten/Lkb-1 deficient mouse model	BEZ235 treatment extended time before endometrial tumor onset and prolonged overall survival	Cheng et al. [41]
Perifosine: AKT inhibitors	Proliferation/apoptosis assay in Ishikawa and HEC-1A cells under estrogen-reduced conditions	Perifosine inhibits cell proliferation and induces apoptosis as a single agent in ishikawa and HEC-1A cells	Block et al. [47]
	Analysis of Perifosine activity alone or in combination with cisplatin in Ishikawa and HEC-1A cells	Perifosine induces growth inhibitory effects as a single-agent, and its effects are enhanced when combined with cisplatin	Engel et al. [54]
MK2206: AKT inhibitors	Study of the activity of MK2206 IN Ishikawa cells expressing progesterone receptor B in in vivo and xenografts assays	MK2206 displayed inhibitory and proapototic effects as a single agent. Combination of MK2206 and progestin showed a synergic anti-proliferative effect in xenograft.	Pant et al. [55]
	Analysis of MK2206 effects in EC patient samples overexpressing GRP78 protein	MK2206 treatment blocks GRP78 expression in EC cells and augments cisplatin-mediated cytotoxicity	Gray et al. [56]

An alternative strategy being evaluated is targeting the specific PI3K P110 isoforms involved in cancer; which, because of the important and differing roles of P110 subunits, have the theoretical potential to block relevant targets more completely. P110α-selective inhibitors, such as INK1117 and NVP-BYL719 have shown preclinical activity in tumor cell lines carrying *PIK3CA* mutations, and are currently in early phase clinical trials. The activity of INK1117 is much lower in PTEN-deficient tumor cells [62]. Another first-class, highly selective inhibitor of PI3K P110-δ isoform: GS-1101 has been used and demonstrates limiting toxicities and broader inhibition profiles [63].

Given the high prevalence of both PTEN deficiency and *PIK3CA* mutation in endometrial cancer, it seems likely that the success of isoform-specific inhibitors in endometrial cancer may be dependent on the determination of the *PIK3CA* and PTEN status of individual tumors.

mTOR Inhibitors

Based on the biological rationale of targeting the mTOR pathway, mTOR inhibitors as a single agent have entered clinical trials for patients with endometrial cancer. mTOR inhibitors either inhibit mTORC1 only or are dual mTORC1/2 inhibitors. mTORC1 inhibitors currently in development assays include Everolimus, Temsirolimus, and Ridaforolimus. Everolimus and Temsirolimus (derivatives from rapamycin) have recently shown antitumoral activity in endometrial cancer cell lines, with greatest sensitivity in cells with *PIK3CA* and/or *PTEN* mutations [53]. In addition, Everolimus reduced progression of endometrial hyperplasia in two different *Pten* knockout models [4, 44] and repressed tumor growth in mice xenograft models harboring endometrial cancer cell lines [62]. Consistent with these results, Ridaforolimus also showed antitumoral activity in endometrial cancer cells and mouse xenograft models, with greatest sensitivity seen in cells with increased phosphorylated or total AKT or loss of PTEN [50]. A possible caution to the use of inhibitors targeting only one mTORC complex is the potential loss of the negative regulatory loop on PI3K/AKT/mTOR pathway activity. Considering this, a second generation of mTOR inhibitors, targeting the catalytic sites of both mTOR complexes, has been developed. In preclinical studies, the mTORC1/2 inhibitors AZD8055 and OSI-027 resulted in growth inhibition in endometrial cell lines and in xenograft mice models [51, 62].

Dual mTOR/PI3K Inhibitors

As expected, single-agent treatment with Rapamycin and its analogs activates a negative feedback mechanism leading to increased formation of the mTORC2 complex, which not only phosphorylates and activates AKT, but also promotes eIF4E phosphorylation, favoring its function in the initiation complex [64]. In order to bypass this problem, and induce the maximal inhibition of this pathway combined targeting of mTOR and PI3K inhibitors has been used. In preclinical trials,

GDC-0980 and BEZ-235 reduced cell growth in several cancer cell lines (including endometrial) and tumor xenograft models more efficiently than single node inhibitors alone [65, 66]. However, in vivo results with BEZ-235 were similar, but not better than those seen with Everolimus [53].

AKT Inhibitors

Even though *AKT* mutations are rare, increased AKT signaling is commonly observed in endometrial carcinomas. AKT inhibitors either compete for the ATP-binding site or inhibit AKT allosterically. A potential caution to targeting AKT is that inhibition may lead to an increased compensatory signaling through AKT-independent PI3K effectors, and the loss of negative inhibition of AKT on its downstream targets may also have deleterious effects. Despite these concerns, the allosteric AKT inhibitors Perifosine and MK2206 showed antitumor activity in preclinical investigations in various cancer cell lines, including endometrial cancer cells. Indeed, Perifosine induced apoptosis in human endometrial cancer cell lines under estrogen-reduced conditions and was more effective than both Everolimus and the EGFR inhibitor Gefitinib [47].

Combining PI3K/AKT/mTOR Inhibitors with Other Therapies

A limitation to the use of PI3K/AKT/mTOR pathway inhibitors in endometrial cancer is the presence of numerous signaling feedback loops and cross-talk between signaling pathways. Thus, combination of PI3K/AKT/mTOR inhibitors with other therapies could improve efficacy.

Given the importance of estrogen signaling in type I endometrial carcinoma and cross-regulation between estrogen receptor and PI3K/AKT/mTOR pathways, combining agents that disrupt both pathways may also result in synergistic antitumoral responses. Indeed, the aromatase inhibitor letrozole in combination with Everolimus showed enhanced inhibition of proliferation and induction of apoptosis in endometrial cancer cell lines [45].

Progestins are a common treatment for women with early stage endometrial cancer who wish to preserve their fertility. Although progestins can be effective in EC treatment, some patients are insensitive to treatment or develop resistance. Resistance to progestins has been shown to result from reduced progesterone receptor expression, which, in turns results from overexpression of EFGR; suggesting that downstream pathways of EGFR could be involved in resistance development. Inhibition of PI3K/AKT/mTOR pathway with LY294002 inhibitor resulted in an upregulation of progesterone receptor expression, diminishing cell growth in progestin resistant endometrial cancer cells, and reversed the resistance to progestin in an endometrial cancer xenograft mice model [62, 67].

Activation of receptor tyrosine kinases (RTKs) stimulates both PI3K/AKT/mTOR and RAS/RAF/MEK pathways, and there is significant evidence to suggest that inhibition of these two pathways may be more effective than targeting either

alone. Although the PI3K inhibitor GCD-0941 decreased tumor growth in xenograft mice harboring FGFR2-mutated endometrial cancer cells, only the combination of GDC-0941 with the MEK inhibitor PD0325901 led to robust tumor reduction [60].

Finally, because activation of the PI3K/AKT/mTOR pathway has also been associated with resistant mechanism to standard cytotoxic agents in EC [68, 69], the combination of these agents with PI3K/AKT/mTOR pathway inhibitors may contribute to a more efficacious therapy. To this regard, combination of Paclitaxel and mTOR1/2 inhibitor has resulted in improved responses in endometrial cancer models [62].

RAS–RAF–MEK–ERK Signaling Pathway

The Mitogen-Activated Protein Kinases (MAPK) are a large family of serine/threonine protein kinases that include the extracellular-signal-regulated kinases (ERK), the c-Jun c-JunNH2-terminal kinases (JNKs), and the P38 MAP kinases. These MAPKs can be considered the final step of different signaling cascades. Each cascade consists of three central kinases: MAPK kinase–kinase–kinase, MAPK kinase–kinase, and the MAPK. Within each of the cascades, the signal is propagated by sequential phosphorylation and activation of MAPKKK, MAPKK, and MAPK. Here, we will focus in the MAPK pathway in which the main MAPKs activated are the ERK class of MAPKs [70].

The ERK signaling pathway is activated by a wide range of extracellular signals such as tyrosine kinase receptors (RTKs), G-protein-coupled receptors, integrins, but also by intracellular signals. The canonical activation of the ERK-MAPK signaling pathway is triggered by the binding of growth factors, such as epithelial growth factor (EGF), to their specific tyrosine kinase receptors. Receptor engagement leads to receptor dimerization that results in receptor autophosphorylation in tyrosine residues in their cytosolic tails. Such tyrosine phosphorylation creates docking sites for a large variety of adapter or signaling proteins that will activate downstream signaling pathways. These proteins vary depending on the activated receptor or the cell type. In most cases, the activation of RTKs is transmitted by several mechanisms to the small GTPase Ras, which subsequently triggers the activation of the MAPK cascade.

There are three cellular *RAS* genes that encode four highly homologous 21 kDa proteins: HRAS, NRAS, KRAS4A, and KRAS4B. KRAS4A and KRAS4B result from alternative splicing at the C terminus [71]. The four RAS proteins are small GTPases that function as molecular switches that can alternate between a GTP-bound "on" state and GDP-bound "off" state. The switch between active and inactive RAS conformations is tightly regulated by guanine nucleotide exchange factors (GEF) that promote GDP dissociation and GTP binding, and GTPase-activating proteins (GAP) that stimulate the intrinsic GTPase activity of Ras to switch off signaling [72–74]. In the case of the EGF receptor, receptor phosphotyrosines are recognized by the adapter protein GRB-2, which in turn, recruits the Guanidine Exchange Factor (GEF) SOS to the receptor. SOS (or other GEF proteins) recruitment and activation causes GDP/GTP exchange of RAS. Once RAS is bound to GTP and active, it triggers

the activation of downstream signaling pathways. The canonical signaling pathway activated by RAS is the cascade of MAPK phosphorylation and activation [70]. This is followed by the sequential recruitment and activation of the cascade of MAPKs: Raf (MAPKKK), MEK (MAPKK), and ERK (MAPK).

The MAPKKKs activated by RAS are a group of three serine/threonine kinases designated as RAFs. There are three different isoforms of RAF, A-RAF, B-RAF, and C-RAF with distinct affinities for both the activator, RAS, and the downstream target MEK. The regulation of RAF kinases is highly complex and is still poorly understood. Apart from RAS, RAF activity is regulated by multiple factors, including phosphorylation/dephosphorylation, conformational changes, or interaction with multiple other proteins [75, 76]. RAF kinase phosphorylates and activates the dual-specificity kinases MAP/ERK kinase (MEK) [77]. In humans there are two highly homologous isoforms of MEK, MEK1 and MEK2 and they are commonly referred as MEK1/2. Once active, MEK1/2 catalyzes the phosphorylation of tyrosine and then threonine of ERKs. In humans, there are two also different ERK proteins: ERK1 and ERK2 that share 84 % homology and many functions. ERKs are also commonly referred as ERK1/2 [78]. Active ERK1/2 will phosphorylate cellular substrates to regulate its function. Upon activation ERK1/2 can phosphorylate over 100 known substrates with diverse functions. Activation of ERK1/2 has been reported to regulate a wide range of cellular processes including proliferation, survival, cell migration, and cell metabolism (Fig. 6.2).

It is worth mentioning that in addition to the canonical MAPK signaling, RAS can activate multiple downstream signaling effectors and pathways such as the

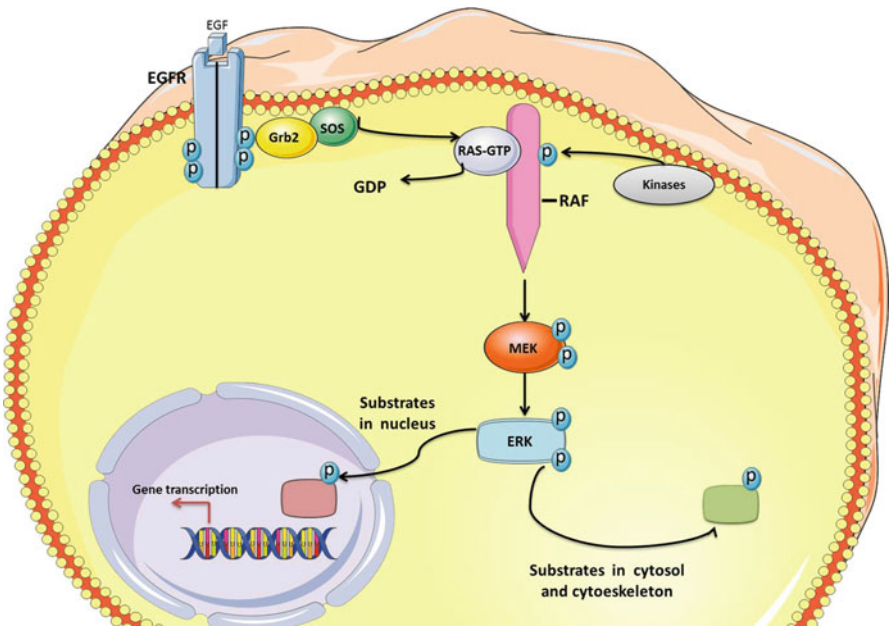

Fig. 6.2 Schematic representation of the RAS-RAF-MEK-ERK signaling pathway

PI3K/AKT, PLCε, RALGDS GTPase, and many more [71, 79]. All these pathways drive different cellular responses to RAS activation and enhance the complexity of RAF signaling.

Alterations in RAS–RAF–MEK–ERK Pathway

The RAS–RAF–MEK–ERK pathway is frequently mutated in human cancers. Most of the mutations in RAS–RAF–MEK–ERK signaling are present in *RAS* and *RAF*.

RAS Mutations

RAS genes were the first oncogenes identified in human cancer cells [80–83]. The key oncogenic mutations are in the region that is identical among the *HRAS, KRAS*, and *NRAS*. Forty-four different point mutations have been characterized in *RAS* isoforms, with 99.2% of them occurring at codons 12, 13, and 61 [84, 85]. All these point mutations are single base substitutions that leads to a constitutive activation of RAS. Although all *RAS* isoforms share the hot spots of mutation, there is a marked difference in the frequency of mutation of each isoform. Among the three *RAS* genes, *KRAS* is the most frequently mutated in human cancers [86, 87]. The Catalog of Somatic Mutations in Cancer (COSMIC, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) database revealed the presence of *KRAS* mutations in 22% of all tumors analyzed, compared with 8% for *NRAS* and 3% for *HRAS*. However, a molecular explanation for why -*RAS* mutation is more frequent in human cancers than -*RAS* or *HRAS* is still lacking. The frequency of mutations of the three *RAS* isoforms varies among tumoral types. *KRAS* is frequently mutated in pancreatic, colon, stomach, endometrial, and lung cancers [87]. In contrast, *HRAS* mutations are present in tumors of the urinary tract and paragangliomas and *NRAS* mutations are preferentially found in melanomas and to a lesser extent in multiple myelomas.

RAF Mutations

Among the members of the RAF family, *BRAF* is the most frequently mutated in human cancers [88]. Genome-wide screens of human cancer demonstrate that *BRAF* is frequently mutated in melanoma, thyroid, lung, and colon carcinomas; in contrast *BRAF*, *ARAF,* and *CRAF* mutations are extremely rare. Functional consequences of excessive signaling participate in several aspects of the tumoral phenotype, such as cell survival, proliferation, cell metabolism, or regulation of the immune response [79].

Alterations in RAS–RAF–MEK–ERK Pathway in Endometrial Cancer

The molecular alterations of RAS–RAF–MEK–ERK signaling are found in the endometrioid type of endometrial carcinomas. In endometrioid endometrial cancer, most of the mutations affecting this signaling pathway are found in *KRAS*. Since the first studies reporting *KRAS* mutations in endometrial cancer [89–91], a large number of mutational analysis confirmed that *KRAS* is frequently mutated in endometrial hyperplasias and carcinomas [92–97]. As in other types of carcinomas, mutations in codon 12 are the most frequent in endometrial carcinomas. The mutational status of *KRAS* and other members of the RAS–ERK signaling pathway have recently been confirmed by an integrated genomic characterization of endometrial cancers [13]. This study performed by The Cancer Genome Atlas (*TCGA*) provided a genome-wide characterization of 373 endometrial carcinomas. In this study, the analysis of a set of 26 different genes involved in the regulation of RAS–RAF–MEK–ERK signaling revealed that 125 samples (52.1 %) were mutated in at least one of these genes. Among these mutations, those affecting *KRAS* were found in 50 out of 240 samples (20.8 %). Thirty-three (60 %) and 9 (18 %) of these mutations were found in codon 12 or codon 13, respectively. In contrast, *RAF1* and *-RAS* displayed low frequency of mutations (2.9 % and 0.4 %, respectively). Regarding the next step in the RAS–ERK cascade, the *RAF* oncogenes, most studies reported an absence or low frequency of mutations in these genes [98–101]. TCGA studies have confirmed these previous data. Only 2.9 % of endometrial carcinomas analyzed displayed *BRAF* mutations. Interestingly, in TCGA none of the mutations identified corresponded to the V600E mutation. In addition to these point mutations, *KRAS* and *BRAF* can display other molecular alterations such as overexpression, gene amplification, or deletions.

Apart from the mutations affecting the core RAS–RAF–MEK–ERK signaling, other molecular alterations in genes involved in the regulation of RAS–ERK signaling have been reported. Promoter hypermethylation of the regulators of RAS–ERK signaling RASSF1A and Sprouty2 [102–106] or overexpression of the scaffold protein KSR1 [107] has been observed in endometrial carcinomas.

The functional consequences and the contribution of RAS–RAF–MEK–ERK alterations to the phenotype of EC are still poorly understood. In vitro, introduction of oncogenic *RAS* in combination with *-RB* inactivation and telomerase activation is sufficient for in vitro neoplastic transformation [108]. In vivo, genetically modified mouse models revealed that, in contrast to other genes such as *PTEN*, *KRAS* mutation is not sufficient to induce endometrial carcinogenesis but can have a synergestic effect with other chemical or genetic tumorigenic insults. Transgenic mice carrying a human prototype *HRAS* gene do not develop endometrial carcinoma; however, a single intraperitoneal injection of the chemical carcinogen *N*-ethyl-*N*-nitrosourea leads to a rapid induction of uterine endometrial proliferative lesions [109]. Similarly, conditional knock-in mice expressing a glycine to aspartate point mutation in codon 12 of *KRAS* (*KRAS G12D*) do not show any pathological altera-

tion in the uterus [110]. However, mice with conditional genetic ablation of *PTEN* and *KRAS G12D* mutation develop invasive endometrioid-type endometrial adenocarcinoma by 4 weeks of age. All these findings support that *KRAS* contributes to neoplastic transformation in the endometrium in the presence of other defined molecular alterations.

RAS–RAF–MEK–ERK Signaling Inhibitors in Preclinical Studies

The high frequency of molecular alterations in the RAS–RAF–MEK–ERK signaling pathway in human cancers prompted an interest in the development of pharmacological inhibitors to target this pathway. Because RAS family members are difficult to target, the development of specific inhibitors has been concentrated on the downstream kinases RAF and MEK [111]. Unfortunately, the current generation of RAF and MEK inhibitors shows very limited therapeutic efficacy as single agents and the mechanisms of resistance remain poorly understood [112].

Although there are an increasing number of inhibitors that target different steps of the RAS–RAF–MEK–ERK pathway that are currently in use for different types of human cancers, few studies have been performed in endometrial cancer [113]. Preclinical studies using RAF or MEK inhibitors have demonstrated null or limited activity as single agents; however, some studies suggest that they can have synergistic activity in combination with drugs targeting other signaling pathways, especially with those targeting the PI3K/AKT signaling pathway [62]. For example, the AN3CA endometrial cancer cell line xenografted in nude mice was insensitive to single-agent treatment with the MEK inhibitor PD0325901 but the combination with the PI3K inhibitor GDC-0941 halted tumor growth [60]. Likewise, other studies demonstrated that combination of the PI3K/mTOR inhibitor BEZ-235 with the MEK inhibitor PD98059 also synergistically suppressed proliferation in endometrial cancer cell lines with *PTEN* and *KRAS* mutations [53]. Future research will be needed to determine whether RAS–RAF–MEK–ERK inhibition may be affective, at least, in combination with other targeted therapies.

Tyrosine Kinases

Tyrosine Kinases (TKs) are a small but relevant subgroup of 90 protein phosphotransferases within the 518 known protein kinases encoded in the human genome [114].

As with all protein kinases, protein tyrosine kinases transfer phosphate groups from high-energy donor molecules to specific receptor substrates (in this case on Tyr residues), inducing substrate conformational changes and thus ultimately regulating target protein function.

Tyrosine kinase family members are categorized into two different groups: (a) receptor tyrosine kinases (RTKs) composed of 58 tyrosine kinases organized in 19 subfamilies and (b) nonreceptor tyrosine kinases, organized in 10 subfamilies.

RTKs are key cell components in sensing and transmitting external stimuli into the cell. They all share a common monomeric structure composed of an extracellular N-terminal ligand binding domain, a transmembrane helix domain, and a C-terminal intracellular domain with tyrosine kinase activity [115]. On the other hand, nonreceptor tyrosine kinases are cytoplasmic, soluble tyrosine kinases that can localize in multiple cell compartments such as the nucleus, cytosol, and the inner surface of the plasma membrane [116]. Upon activation, nonreceptor tyrosine kinases propagate and execute intracellular communication that finally result in the cellular response to stimuli.

As signaling molecules, tyrosine kinases have been shown to play leading roles in the development of multiple diseases, including cancer [117]. In this regard, in recent years structural and functional studies have pointed to tyrosine kinases as essential components of these processes by mediating and participating in multiple biological functions, for example, cell proliferation, negative regulation of apoptosis or angiogenesis [118, 119]. Moreover, these functions are often perturbed during tumor progression as a consequence of a hyperactive state of the tyrosine kinases. Therefore, tyrosine kinases are frequently considered prototypic oncogenes.

The emergence of high-throughput and *omics* technologies has led to the discovery of novel alterations in TKs, such as the presence of activating mutations [120, 121] or increased expression due to genomic amplifications [122–124] suggesting that cell-autonomous activation of TKs may drive transformation. Tyrosine kinases have been shown to be ideal targets for anticancer therapy in vitro and in vivo in preclinical models (Table 6.2) and, in some cases, have looked promising in clinical trials.

Alterations in Receptor Tyrosine Kinases in Endometrial Cancer

RTKs play a prominent role in regulating development and progression of EC. Indeed, multiple members within several of its different subfamilies have been shown to participate in the multifaceted progression of EC, from tumor growth to angiogenesis, to dissemination and distant organ colonization.

EGFR Family

One of the first RTK families implicated in EC was the epidermal growth factor receptor family (EGFR), which is known to play critical roles in cell growth and differentiation. The epidermal growth factor family is comprised of EGFR (ErbB1), HER2/Neu (ErbB2), HER-3 (ErbB3), and HER-4 (ErbB4). EGFR and HER-2/Neu have been shown to be highly expressed in normal endometrium and overexpressed in EC, where they have been associated with a poor prognosis [138, 139], and to regulate cell invasion, growth, and apoptosis [127, 140–142]. Also, overexpression

Table 6.2 Preclinical studies using TK inhibitors currently under clinical trials

Compound	Experimental approach	Effect	Ref
Gefitinib (EGFR)	Effects of reconstitution of Rb and PTEN in Ishikawa cells and correlation with Gefitinib activity	Sensitiation to pro-apoptosic effects of Gefitinib	Albitar et al. [125]
	Analysis of growth effects by treating 6 EC cell lines with Gefitinib in combination with selected anticancer agents	Growth inhibitory effects and synergistic cytotoxic effects when combined with Doxitacel and Paclitaxel	Gaikwad et al. [126]
	Proliferation/apoptosis assays of Gefitinib under estrogen-reduced conditions	Gefitinib inhibited cell proliferation alone, and showed synergistic anti-proliferative effects with Everolimus	Block et al. [47]
	Analysis of gefitinib effects in vivo using Ishikawa cells xenografts	Gefitinib overcomes resistance to progestin resistance in the progestin-resistant Ishikawa-pLWERNL subcell line	Xu et al. [127]
Erlotinb (EGFR)	Analysis of Erlotinib effects on the MUC20-enhanced invasive phenotype	Impairment of MUC20-STAT3-induced cell migration and invasion	Chen et al. [128]
Cetuximab (EGFR)	In vivo/in vitro effects of Cetuximab in HEC-1A xenografts	Cetuximab displayed cell proliferation inhibitory effects in vitro and blocked tumor growth and dissemination in vivo	Takahashi et al. [129]
Trastuzumab (ErbB2)	Correlation of trastuzumab actions with PTEN expression in EC cells.	Decreased PTEN expression is associated with increased resistance to Trastuzumab	Pfeiler et al. [130]
	Analysis of estrogen effects in Trastuzumab-treated cells	Estradiol counteracts Transtuzumab cytotoxic activity through activation of ERK1/2	Treeck et al. [131]
	Analysis of Pertuzumab activity alone or in combination with Trastuzumab in uterine serous EC cell lines	Trastuzumab induces antibody-dependent cell-mediated cytotoxicity (ADCC) and its effects are enhanced when combined with complement-containing plasma and interleukin-2 or with Pertuzumab	El-Sahwi et al. [132]
	Analysis of CD46, CD55 and CD59 function in cell response to Trastuzumab in uterine serous carcinoma (USC) cells	siRNA inhibition of CD55 and CD59, but not CD46 potentiates the effects fo trastuzumab in overexpressing Her2/neu USC	Bellone et al. [133]

(continued)

Table 6.2 (continued)

Compound	Experimental approach	Effect	Ref
Bevacizumab (VEGF)	Effects of VEGF/VEGFR inhibition in a in vivo orthotopic mouse model using Ishikawa/ HEC-1A cells.	Combination of bevacizumab with docetaxel decreases tumor progression and vasculature density	Kamat et al. [134]
Brivanib (VEGFR2/FGFR1)	Study of the activity of Brivanib in tamoxifen-stimulated endometrial tumors	Brivanib impairs tumor growth of the tamoxifen-stimulated EnCa 101 endometrial tumors	Patel et al. [135]
Imatinib (c-Kit, PDGFR, Abl)	Isolation of CD117(+) cells from cell culture. Use of anti-SCF antibodies and pharmacological inhibition of CD117 using Imatinib.	Inhibition of Ishikawa and MFE280 c-Kit(+) cancer cells resistance to cisplatin	Zhang et al. [136]
	Analyze the synergistic effect of Imatinib mesylate, lithium chloride and medroxyprogesterone acetate in Ishikawa cells	lithium chloride and medroxyprogesterone potentiate the anti-tumor effect of Imatinib by inhibiting cell proliferation and activating caspase-3,	Bilir et al. [137]

Table 6.3 Main EC inhibitors used in published clinical trials

Compound	Target	Clinical trial phase	Prior chemotherapy	No of patients	Response rate (%)	Ref
Temsirolimus	mTOR	Phase II	No	33	14	Oza et al. [152]
		Phase II	Yes	27	4	
Ridaforolimus	mTOR	Phase II	Yes	45	11	Colombo et al. [153]
Everolimus	mTOR	Phase II	Yes	44	9	Ray-Coquard et al. [154]
Erlotinib	EGFR	Phase II	No	34	12.5	Oza et al. [151]
Gefitinib	EGFR	Phase II	Yes	29	3.8	Leslie et al. [149]
Trastuzumab	ErbB2	Phase II	Yes	33	0	Fleming et al. [148]
Lapatinib	HER2	Phase II	Yes	30	3	Leslie et al. [155]
Bevacizumad	VEGFR	Phase II	Yes	56	13.5	Aghajanian et al. [156]
Brivanib	VEGFR2 /FGFR1	Phase II	Yes	45	18.6	Powell et al. [157]
Sorafenib	Multi-TRKs	Phase II	Yes	56	5	Nimeiri et al. [158]
Sunitinib	Multi-TRKs	Phase II	Yes	34	18	Castonguay et al. [159]
Temsirolimus +bevaciz umab	mTOR/ VEGF	Phase II	Yes	53	24.5	Alvarez et al. [160]
Everolimus +letrozole	mTOR/ Aromatase	Phase II	Yes	38	15	Slomovitz et al. [161]

due to genomic amplifications and also point mutations in *EGFR* locus has been found in endometrial carcinosarcomas [143].

Overexpression of ErbB3 and ErbB4 has also been observed in endometrial tumors by immunohistochemistry and gene expression profiles [144, 145]. More recently, an integrated systems biology approach consisting of whole-exome sequencing coupled with loss of-function screenings uncovered *ERBB3* as a driver cancer gene in EC, although its functional role in endometrium still remains unclear [145–147]. The pivotal role of EGFR and ErbB2 in the progression of endometrial cancer has received significant attention and, as a result, several inhibitory compounds are in clinical trials [148–151] (Tables 6.2 and 6.3).

VEGFR Family

Vascular endothelial growth factor (VEGF) family members have been long linked to tumorigenesis due to their role in promoting angiogenesis and hence supplying cancer cells with oxygen and nutrients. Therapeutic strategies based on targeting VEGF-related proteins have potent antitumoral effects in preclinical models and in

the late 1990s anti-VEGF molecules were tested in clinical trials for cancer patients [162]. In endometrial cancer, several VEGF members show increased expression that has been linked with poor outcome. In particular, VEGF-A and VEGF-D and their cognate tyrosine kinase receptors VEGFR1 (Flt-1), VEGFR2 (Kdr), and VEGFR3 (Flt-4) have been found overexpressed in three independent series of 115, 71, and 76 endometrial cancer specimens [163–166]. Despite contradictory results in some cases, it is generally thought that immunoreactivity for these proteins increased as lesions progressed from normal endometrium to advanced carcinoma and correlated with microvessel density, tumor grade, stage, lymphovascular infiltration, metastasis, and increased risk for poor outcome. In preclinical studies, inactivation of VEGF receptors using the anti-VEGF agent Bevacizumab has shown great effectiveness against endometrial cancers cells in orthotopic mouse models with associated decreased proliferative potential and microvasculature density [134]. Bevacizumab and Brivanib (a specific VEGFR2/FGFR1 pharmacological inhibitor) are currently being tested in Phase II clinical trials for patients with advanced or recurrent disease [156, 157].

PDGFR Family

The platelet-derived growth factor receptor (PDGF-R) family is one of the most prominent and large RTK families containing multiple members that are altered in endometrial cancer.

The platelet-derived growth factor receptor (PDGF-R) isoforms α and β are the cognate receptors for PDGF ligands. The PDGF/PDGF-R system is involved in cell differentiation, migration, and tissue remodeling during normal development and in normal adults [167]. It also controls proliferation, motility, and contractility of endometrial stromal cells necessary for endometrial tissue repair [168] and fosters tumor growth and invasion of endometrial cancer cell lines [169, 170]. In addition, increased activity of PDGF/PDGF-R by analysis of PDGF-D expression has been associated with myometrial invasion and lymphatic vascular space invasion in endometrial cancer [170]. Also, PDGFRα was expressed in recurrent endometrioid endometrial carcinoma in one study [171] and in another study cytoplasmic and nuclear PDGFRα and β were expressed in uterine sarcomas when compared to normal myometrium or endometrium. Both have been postulated as potential therapeutic targets [143, 172–174].

The proto-oncogene C-Kit (*CD117*) plays important roles during cell differentiation and tissue morphogenesis [175, 176] and its activation upon stem-cell factor (SCF) ligand binding triggers cell proliferation in several types of tumors such as breast and small-cell carcinoma of the lung [177, 178]. Two studies have shown C-Kit positive immunostaining in 58 % and 30 % of endometrial adenocarcinomas in two independent cohorts of 72 and 10 endometrial adenocarcinomas, respectively. Positivity for C-Kit correlated with myometrial invasion, metastatic potential, and decreased disease-free survival [179, 180]. Interestingly, in vitro targeted therapy against C-Kit reduced the proliferative capacity, colony formation in soft agar, and resistance to cisplatin in Ishikawa and MFE280 C-Kit(+) endometrial can-

cer cells [136]. In addition, C-Kit increased expression and mutations have been observed in gynecologic carcinosarcomas [143].

Colony-stimulating factor 1 receptor (CSF-1R), the product of the *C-FMS* proto-oncogene, is the canonical receptor for colony-stimulating factor 1 (CSF-1), a well-known regulator of phagocyte proliferation and differentiation. In addition, CSF-1/CSF-1R is important during pregnancy as their activity increases in uterine epithelium, preimplantation embryos, decidual cells, and trophoblasts [181–186]. CSF-1R was one of the first RTK found overexpressed in endometrial adenocarcinoma and correlated with high grade, advanced stage, and poor prognosis [187–190]. On the contrary, CSF-1R is not involved in development and progression of uterine sarcomas [191].

INSR Family

The insulin receptor (IR) is one of the most investigated RTKs to date. Its two isoforms (IR-A and IR-B) share distinctive functional and biological properties. While IR-B is a classical receptor that regulates glucose uptake, IR-A presents higher affinity for insulin growth factor-2 (IGF-2) [192, 193], has potent mitogenic and antiapoptotic effects, and is found overexpressed in many tumor types including endometrial cancer [194–196].

The insulin growth factor 1 receptor (IGF-1R) is a tyrosine kinase receptor that binds IGF1 and IGF2 and signals through the activation of the insulin receptor substrate family of proteins (IRS) and the PIK3/AKT/mTOR pathway [197]. IGF-1R is widely expressed in normal and neoplastic tissues and in the endometrium it localizes in the luminal, glandular epithelium, and the stroma. Interestingly, both IGF1 and IGF1R are transcriptionally regulated by estrogen in normal endometrium and endometrial cancer cells and stimulate cell proliferation [198–201].

Despite the fact that alterations at a DNA level are infrequent, increased levels of IGF1R have been observed in cancers including those from the endometrium [202, 203]. In regards to endometrial adenocarcinoma, overexpression of IGF-1R at the RNA level [204] and increased phospho-activated IGF-1R and downstream p-AKT have been detected compared to normal proliferative endometrium [202, 205]. It has been proposed that this pathway contributes to the risk of endometrial hyperplasia and cancer. Finally, inhibition of IGF-1R activity through multiple strategies such as interference RNA, pharmacological inhibition, or the use of therapeutic antibodies dampens endometrial cancer cell proliferation and restores sensitivity to chemotherapy [206–209].

The anaplastic lymphoma kinase (*ALK*) gene, which encodes a tyrosine kinase receptor that belongs to the insulin receptor superfamily, is frequently altered in anaplastic lymphomas and nonsmall cell lung cancer (NSCLC) [210, 211]. Alterations at the DNA level involve mainly chromosomal rearrangements causing activation of the receptor and downstream targets such as AKT, STAT3, and MAPK, finally resulting in cell proliferation, differentiation, and antiapoptosis [210–213]. Recently, additional alterations in *ALK* seen in NSCLC such as mutations and amplifications have been found to provide resistance to tyrosine kinase inhibitor

(TKI) therapy [214–216]. *ALK* alterations have not been extensively studied in EC. However, amplifications have been observed at low frequency (1.3 %) in endometrial carcinosarcomas [143].

MET Family

Hepatocyte growth factor ligand (HGF) signals through the mesenchymal epithelial transition factor (MET) tyrosine kinase receptor (also known as hepatocyte growth factor receptor/HGFR). Both factors have been found overexpressed in various tumor types where they regulate motility, angiogenesis, cell growth, and colonization in new environments [217–221]. HGF/MET axis activates an intracellular signal cascade initially involving the adaptor proteins GAB-1, GRB-2, and SHC that ultimately trigger the activation of several transduction pathways such as PI3K, FAK, or STATs [222].

In endometrial cancer, C-Met protein expression is higher when compared to atrophic endometrium and has been correlated with surgical stage III and IV, histologic Grade 3, and poor survival [223, 224]. Recent studies indicate that HGF/C-MET signaling promotes migration and anoikis resistance by inducing the expression and activity of MMP-2 and MMP-9 and by increasing the expression of cyclooxygenase-2 through a PIK3/AKT-dependent mechanism, respectively [225–227]. Finally, *MET* has been found mutated in endometrial carcinosarcomas resulting in alterations at residues R970 and T992 although the relevant implications for these sequence variants are still unknown [143].

FGFR Family

The fibroblast growth factor (FGF) signaling pathway is fundamental in proliferation and differentiation during embryogenesis and in adult tissue homeostasis [228, 229]. Its multiple and broad effects are cell and tissue type dependent and its effects are contextualized by a large number of members that include 18 FGF ligands and 4 conserved fibroblast growth factor receptors (FGFRs) [230].

The FGF/FGFR pathway is altered in several types of cancers due to genetic alterations including activating mutations, gene amplification/overexpression, and chromosomal translocations [228, 230, 231].

In endometrium, the FGF/FGFR system contributes to its normal physiological function in various phases of the menstrual and estrus cycles [232–236] but has also been found altered in pathological conditions such as cancer. In particular, alterations in FGFR2 are more common than other family members in endometrial carcinoma. Recent advances point to activating mutations as the main genetic alteration in a significant proportion of endometrial cancers (10–16 %) [120, 232, 237, 238] resulting in constitutive receptor dimerization or increased ligand-receptor affinity [239–241]. Nonetheless, its association with prognosis is unclear. Results coming from sequencing-directed mutational analysis as well as pharmacological/interfer-

ence RNAi inhibition indicate that, in endometrial cancer, FGFR2 mutations foster tumorigenesis mainly through the MAPK pathway [120, 242].

EPH Family

The ephrin receptors (EPHR) are split into two different groups, EPHA and EPHB, according to their molecular structure and affinity for the ligands ephrin-A and ephrin-B. The EPH/EPHR signals are essential for proper vasculogenesis and organogenesis [243–245, 353, 369] and have been recently observed to play key roles in endometrial multipotent mesenchymal stromal cells (MSC) during early stages of regenerative adult neovascularization [360].

Immunohistochemical studies performed in a series of 139 and 20 endometrial cancer cases have revealed increased protein expression of EPHB4 and EPHA2, which correlated with several clinicopathological parameters such as tumor stage, grade, and depth of myometrial invasion [294, 355]. More recently, EPHA2 has been postulated as a predictive biomarker of poor prognosis in endometrial cancer and a suitable therapeutic target as the use of the EPHA2-agonist monoclonal antibody EA5 has proven antitumor properties in vivo using orthotopic mouse models of uterine cancer [317].

Nonreceptor Tyrosine Kinases in Endometrial Cancer (NRTKs)

Unlike RTKs, fewer studies have dissected the contribution of NRTKs in endometrial cancer. NRTKs, or cytoplasmic tyrosine kinases, are crucial factors that transmit and articulate extracellular signals often sensed by transmembrane receptors. Biologically, NRTKs act as central hubs participating in critical cellular functions such as differentiation, survival, and proliferation. Not surprisingly, alterations involving NRTKs contribute to tumorigenic processes and several cytoplasmic tyrosine kinase inhibitors are under study for therapeutic applications.

SRC Family

The sarcoma (SRC) group of proteins is the largest family of NRTKs and participate in a broad spectrum of cellular functions such as survival, migration, and differentiation [277, 312, 358].

SRC, the first retroviral oncogene to be identified, has been found altered in many types of cancer such as colon, breast, melanoma, and lung, where it has a relevant role in promoting tumorigenesis [266, 278, 281, 377]. In contrast, in endometrial cancer either total SRC or phospho-active SRC are not associated with progression from normal to malignant endometrium or with any clinicopathological parameter analyzed to date [259, 271].

FAK Family

The focal adhesion kinase (FAK) localizes to adhesions between cells and extracellular matrix and conducts the signal cascades that derive from these interactions, especially from integrins [262, 344]. FAK participates in tumor progression [320, 357, 366] and in endometrial cancer has been found overexpressed by immunohistochemistry when compared to normal endometrium. Its overexpression has been correlated to histological grade, P53 overexpression, myometrial invasion, cervical involvement, and lymphatic vascular space invasion [282, 309, 380]. Recently, it has been demonstrated that FAK is essential for estrogen and tamoxifen-derived promitogenic actions and that FAK regulation at the posttranscriptional level by microRNAs dampens proliferation, migration, and invasion of endometrial cancer cells [256, 362].

JAK Family

The janus kinase family consists of four members: JAK1, JAK2, JAK3, and TYK2. While JAK3 is preferentially expressed in the hematopoietic tissues and lymphoid precursor cells, JAK1, JAK2, and TYK2 are expressed ubiquitously [279, 289, 328, 373]. JAKs are activated by cytokines, signal through the STAT family of proteins, and are critical mediators of inflammation, hematopoiesis, and immunity. Also, JAK/STAT deregulation has been observed in myeloproliferative neoplasms (MPNs), autoimmune disorders, and immunodeficient conditions. In particular in MPNs, increased JAK/STAT activity has been linked to activating mutations in JAK2 [299, 305, 365].

In endometrial cancer, however, recent findings suggest that truncating mutations affecting the kinase domain of *JAK1* take place frequently causing a loss-of-function phenotype. These alterations are thought to contribute to tumor immune evasion [335].

WNT/β-Catenin Signaling Pathway

The WNT/β-catenin pathway plays a pivotal role in cell biology controlling various cellular processes such as cell proliferation, differentiation, and maintenance of pluripotency [274]. The activation of WNT signaling is involved in many cancer types, underscoring the importance of this pathway in controlling different aspects of cancer biology [263]. β-catenin/Armadillo, is a multifunctional protein of 92 kDa, that interacts with the intracytoplasmic region of E-cadherin maintaining epithelial cell integrity. It is also the key downstream effector of the WNT/Wingless pathway, also referred to as WNT/β-catenin or “canonical” WNT signaling pathway [287]. In the absence of WNT signal activation, a large protein complex, which is composed of the scaffolding protein Axin-1/-2, the tumor suppressor adenomatous polyposis coli (APC), casein-kinase1 (CK1), disheveled and

glycogen synthase kinase 3 GSK-3β, phosphorylates β-catenin at serine/threonine residues near the NH3 terminus, inducing its degradation through the ubiquitin proteasome pathway [329, 371]. When WNT ligands bind to a coreceptor complex formed by a transmembrane frizzled receptor and a low-density lipoprotein receptor-related protein 5 or 6, it results in the canonical activation of the WNT receptor, leading to the inhibition of Axin and GSK-3β, which hampers beta catenin breakdown and induces its accumulation [371, 376]. Hypophosphorylated β-catenin is stabilized and enters the nucleus where it interacts with the T-cell factor (TCF)/Lymphoid enhancer family (LEF) family of transcription factors, leading to transcriptional activation of specific target genes. The canonical WNT signaling target genes include Cyclin D1, C-MYC, and MMP-7, which promote cell survival, cell cycle progression, and uncontrolled proliferation [265, 292, 322, 356]. Mutations in components of the WNT cascade, such as APC or β-catenin lead to an aberrant activation of WNT pathway, and are often associated with tumor growth and metastasis [336, 346, 354]. Of note, WNT ligands can also activate other downstream signaling pathways that act independently of β-Catenin. This pathway is referred to as the "noncanonical" WNT pathway and involves the activation of different signaling cascades such as protein kinase C (PKC) and c-Jun N-terminal kinases (JNK). β-Catenin independent WNT signaling pathway has been shown to control the biology of different types of tumors [298].

Alterations in WNT/β-Catenin Pathway in Endometrial Cancer

WNT/CTNNB1 signaling pathway is frequently activated in type I endometrial carcinoma. *CTNNB1* mutations have been detected in endometrial hyperplasias, suggesting that these mutations occur in the early stages of the neoplastic process [315]. Activating mutations in exon 3 of the *CTNNB1* gene were identified in the late 1990s and were shown to consist of missense mutations in one of the serine/threonine residues. These mutations affect codons 41, 45, 33, and 37 and alter the phosphorylation consensus motif of GSK-3β, hampering GSK-3β-mediated β-Catenin degradation [297, 315]. Although mutations or deletions in the *CTNNB1* gene seem to be the most common mutational event that affects the WNT pathway in EC, alternative mechanisms, such as epigenetic silencing of WNT antagonists have been shown to regulate this pathway in EC. For instance, although no mutation in the sequence of *APC* was found in EEC [345], its expression was found to be decreased. In fact, the Yin Yang1 (YY1) transcription factor, that has been shown to be overexpressed in EEC, silences APC expression through an epigenetic mechanism that involves the recruitment of the Histone-lysine N-methyltransferase enzyme EZH2 and the trimethylation of histone 3 lysine 27 on its promoter region [375]. Moreover, the protocadherin PCDH10, shown to be down regulated in EEC, has been implicated in inhibiting the WNT/β-catenin signaling pathway in EEC [379]. Recently, mutations in RNF43, the E3 ubiquitin ligase that negatively regulates WNT signaling have been detected in 18% of colorectal adenocarcinomas and endometrial

carcinomas, and have been found to prevail in microsatellite-unstable tumors [285]. The SOX7 transcriptional factor, whose expression is downregulated in EC, has been shown to negatively regulate the WNT pathway in EC through impeding the transcriptional machinery of β-Catenin/TCF/LEF-1 [257]. The WNT pathway has also been shown to be involved in cross talk with other signaling pathways such as mTOR and Hedgehog, and to control estrogen and progesterone signaling pathways in EC [269].

WNT/β-Catenin Signaling Inhibitors in Preclinical Studies

The WNT oncogenic pathway, activated in many cancers including EC, seems a highly attractive target in cancer, as this pathway is crucial for the maintenance of tumor-initiating cells. Unfortunately, the development of WNT pathway inhibitors is still at an early phase and far from clinical trials. Several causes have been attributed to explain this delay. In cancer models, the redundancy in WNT ligands (19 known Wnt ligands), and FZD receptors isoforms (10 FZD isoforms), renders this pathway very difficult to target therapeutically. Moreover, it has been suggested that since WNT pathway controls early tumorigenic events, it induces irreversible differentiation of cancer cells that no longer respond to WNT inhibition. However, recent work shows that the benzopyran compound 2-(piperidinoethoxyphenyl)-3-(4-hydroxyphenyl)-2H-benzo (b)pyran(K-1), a potent antiestrogenic agent, induces apoptosis in endometrial hyperplasia, by inhibiting both the WNT and PI3K/AKT/mTOR pathways [258]. Moreover, other compounds targeting WNT ligands, frizzled receptors or β-Catenin, have given promising results in vitro and in vivo preclinical models: OMP-18R5 is a therapeutic monoclonal antibody that interacts with five Frizzled receptors, blocking their activity. OMP-18R5 has been shown to inhibit the growth of various patient-derived xenografts [288]. The soluble WNT decoy receptor OMP-54F28 has also been tested in preclinical models and has shown reduced tumor growth and decreased numbers of CSCs (cancer stem cells). This compound is actually undergoing 3 phase 1b studies in ovarian, pancreatic, and hepatocellular cancers [302]. Another compound, that has been shown to potently inhibit WNT signaling in vitro and in vivo, is LGK974, an inhibitor of WNT ligand secretion. LGK974 has demonstrated to be effective in breast cancer models and a head and neck squamous cell carcinoma model [308]. PRI-724 is a second-generation-specific CBP/Catenin antagonist. In a phase I study using PRI-724 in patients with solid tumors, PRI-724 showed acceptable toxicity profile and induced a decrease in the expression of the biomarker survivin [304]. PRI-724 is currently in clinical trials for advanced myeloid malignancies and advanced solid tumors.

Targeting WNT pathway has also been achieved using inhibitors that block the WNT signaling in the nucleus. PKF115-584, CPG 049090 are antagonists of TCF/β-Catenin complex and have shown to decrease the number of invasive endometriotic epithelial cells of patients with endometriosis [316]. These compounds have also demonstrated the ability to inhibit cell growth in different cancer models such as HCC [370], lymphocytic leukemia [283], and colorectal cancer [321].

Tankyrase inhibitors have also emerged as possible WNT inhibitors. Inhibition of tankyrase activity promotes Axin stabilization, reducing WNT pathway activation. XAV 939 inhibits cell migration in breast cancer [250], while it induces apoptosis in neuroblastoma [359]. Moreover, the novel tankyrase inhibitor JW55 showed promising results in CRC (colorectal cancer), inducing a reduction of tumor growth [368].

In endometrial cancer, the WNT pathway is complex, as it is linked to crucial pathways controlling endometrial cell growth. To date, few studies have addressed the effects of WNT inhibitors in EC. Recent results have however suggested a role of this pathway in controlling EC growth in vitro and in vivo [268]. Future research is needed to address the safety and the therapeutic benefit of Wnt-targeted therapy in patients with EC.

Cell Cycle

Most eukaryotic cells undergo a cell cycle composed by four differentiated phases (G1, S, G2, and M phases). This process is controlled by three major checkpoints (located in the transition from G1 to S phase, G2 to M phase, and during M phase in the transition from metaphase into anaphase), which govern the safe and accurate replication of their genomes [272]. Although most of the checkpoint-sensing mechanisms are still unclear, they seem to converge on two sets of proteins that act together to trigger cell cycle advancement: the Cyclins—A (A1, A2), B (B1, B2, B3), D (D1, D2, D3), and E (E1, E2)—and the cyclin-dependent kinases (CDK 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and CDK-activating kinase) [311]. Both Cyclins and CDKs are families of related proteins and combine in different ways to form specific Cyclin-CDK complexes that govern particular points in the cell cycle. Interestingly, the intracellular level of CDKs is fairly constant, while the level of Cyclins fluctuates dramatically depending on the state of the cell with respect to the cell cycle. The Cyclins are proteins that regulate progression through the cell cycle and must be present in sufficient concentration to help activate the appropriate CDK. The CDKs are serine/threonine kinases and compose the active, enzymatic, half of the partnership, which activate other enzymes by phosphorylation. Although the Cyclins appear to be necessary for CDK activation, they are not sufficient. There are intermediate phosphorylation and dephosphorylation steps, and fluctuation of CDK inhibitors (CKIs), that are required to activate the CDK after Cyclin binding. There are two CKIs families: the INK4 inhibitors and the CIP/KIP inhibitors, with four members—P16INK4A (P16), P15INK4B (P15), P18INK4C (P18), and P9INK4D (P19); and the CIP/KIP family, with three members—P21Waf1/Cip1 (P21), P27Kip1 (P27), and P57Kip2 (P57). The INK4 family inhibits CDK4 and CDK6 activity during G1 phase specifically, whereas the CIP/KIP family can inhibit CDK activity during all phases of the cell cycle [350]. Levels of CKIs, which specifically inhibit certain Cyclin/CDK complexes, also rise and fall at specific times during the cell cycle.

In G1, which is the growth phase, activation of Cyclin D–CDK4/6 is responsible for G1 progression. This complex phosphorylates the tumor suppressor

Retinoblastoma protein (RB) and subsequently, Cyclin E is synthesized. The complex Cyclin E–CDK2 is necessary for the G1-S transition. As part of this process, activated CDK2 promotes further phosphorylation of RB, which then dissociates from E2F, allowing E2F to activate the transcription of genes required for S phase. E2F activity consists of a heterodimeric complex of an E2F polypeptide and a DP1 protein. One of the genes activated by E2F is Cyclin-E itself, leading to a positive feedback cycle to promote accumulation of Cyclin-E [264, 275].

Following G1, the next phase of the cell cycle is the S phase [254], during which synthesis of new DNA occurs and results in genome duplication. The Cyclin A–CDK2 complex plays a key role in initiation of replication by activating the prereplicative complex. It also phosphorylates CDC6, causing it to dissociate from the Origin Recognition Complex (ORC), a multisubunit DNA binding complex (6 subunits) that binds to origins of replication in an ATP-dependent manner in all eukaryotes. This process serves as the foundation for assembly of the prereplication complex (pre-RC), which includes CDC6, TAH11, and the MCM2–MCM7 complex. This prevents immediate reuse of this origin of replication, and since the phosphorylation of CDC6 allows it to be recognized by an ubiquitin ligase complex, it is tagged for proteolysis. During G2, CDK1 is maintained in an inactive state by the kinases WEE1 and MYT1 [253]. As cells approach M phase, the phosphatase CDC25 is activated by PlK. CDC25 then activates CDK1, the major mitotic kinase MPF (M phase Promoting Factor) is formed, and finally, the cell proceeds to M phase [296]. The M phase consists of prophase, metaphase, anaphase, and telophase. In prophase, the MPF phosphorylates microtubule motor proteins, and microtubule associated proteins (MAPs) alter the normal microtubule dynamics, to allow the massive reorganization into a mitotic spindle. Metaphase is reached when sister chromatids are lined up along the midline of the mitotic spindle. Before going through anaphase [251], MPF must be inactivated. Deactivation of MPF is also a tightly controlled process. Basically, MPF phosphorylates CDC20 and hence, anaphase promoting complex (APC) is activated. APC is an ubiquitin ligase (type E3) that polyubiquitinates Cyclin B of the MPF complex, making it a target for proteolytic degradation by a proteasome. Activation of APC is also needed to separate the sister chromatids and pull them toward opposite poles of the mitotic spindle. When both sets of chromosomes arrive at their respective poles, telophase begins. Inactivation of APC impairs its ability to phosphorylate nuclear lamins, and consequently, unphosphorylated lamins are able to interact with each other, reconstituting the nuclear lamina and the nuclear envelope. By the end of telophase, cytokinesis splits the cell into two separate and independent daughter cells.

Cell Cycle and Endometrial Cancer

A breakdown in the regulation of the cell cycle leads to uncontrolled growth and contributes to the development of many neoplasias. Probably, the most important gene related to cell cycle and cancer has been *TP53*, which is implicated in G1 cell cycle arrest following DNA damage and in apoptosis when triggered under certain

conditions. In endometrial cancer [293], *TP53* mutations affect more often nonendometrioid cancers (93–100 % of serous type) and 17–61 % of endometrioid cancers. Mutations in *TP53* are associated with statistically significant shorter patient survival [301, 334].

In endometrial cancer, ambiguous results in relation to cell cycle markers have been described. While several authors have reported significant associations between cell-cycle expression and endometrial tumor characteristics, others have not been able to associate those with most of the established risk factors for endometrial cancer, i.e., age, menopausal status, menopausal hormone use, smoking status, body mass index, parity, oral contraceptive use, and stage and grade of the disease.

On one side, overexpression of Cyclin A [342], Cyclin D1 [351], Cyclin E [318], and B1 [343] have been associated with a less differentiated phenotype and advanced stage. High levels of Cyclin E, CDK2, and CDK4 correlate with weak/absent ER expression [319]. In EC, correlations between Cyclins E and A and P53 have been observed [319, 351], as well as correlation of Cyclin E with pRB [319]. Cyclin D1 expression was highly correlated with CDK4 and Ki-67 [300] and was related to the development of a small number of USC cases [347]. In relation to CKI, some authors have reported that overexpression of P16, P21, or P27 is significantly associated with poorly differentiated tumors, advanced stage, serous or clear cell histologies, and worse survival among endometrial cancer patients [318, 340, 351]. Interestingly, P16 and P21 overexpression are significantly associated with low PR immunoreactivity [318].

On the other side, Felix et al. [276] recently showed that CDK inhibitors P16, P21, and P27 were minimally associated with epidemiologic risk factors for endometrioid endometrial cancer. As well, Semczuk et al. [348] demonstrated that neither cell-cycle regulators nor the frequency of pRb, P16, and Cyclin D1 abnormalities were associated to clinicopathological variables of EC, except for CDK4 expression, which was related to clinical stage of the disease. However, 69 % of EC showed abnormal expression of at least one RB-pathway protein immunohistochemically.

Therapeutic Strategies Related to Cell Cycle Pathway

Targeted therapies directed against cell cycle regulators have been difficult to translate into the clinic. However, small-molecule CDK inhibitors are currently being pursued for therapeutic uses in different neoplasias. Early efforts to block CDKs with nonselective CDK inhibitors led to little specificity and efficacy but apparent toxicity; however, the recent advance of selective CDK inhibitors (particularly for both CDK4 and CDK6) allowed the first successful efforts to target these kinases for several diseases therapies [248, 261]. In endometrial cancer, CDK inhibitors have not yet been tested, but other molecules have arisen as possible new targets for therapy. Umene et al. [364] highlighted the importance of targeting Aurora kinase A (AURKA), which regulates the cell cycle checkpoint and maintains genomic integrity, to control endometrial carcinogenesis. In this study, AURKA was associated

with tumor grade and poor histological differentiation. Inhibition of AURKA by interference RNA (siRNA) decreased cell growth, invasion and migration of Hec1B cell lines, and increased chemosensitivity to paclitaxel. Moreover, combination of AURKA siRNA and paclitaxel resulted in a more significant decrease of tumor volume in xenografts assays compared to treatment with paclitaxel only. Further research on targeted cell cycle therapy is needed in endometrial carcinoma.

TGF-β Signaling Pathway

Transforming growth factor β (TGF-β) is the prototype of a large family of secreted polypeptide growth factors (cytokines). To date, up to 33 TGF-β related genes have been identified, including Bone Morphogenic Proteins (BMPs), Activin/Inhibitin, and growth and differentiation factors, Nodal and anti-Müllerian hormones. These cytokines can induce a broad range of cellular responses such as cell proliferation, differentiation, migration, apoptosis, or extracellular matrix production [314, 352]. In terms of carcinogenesis, TGF-β is a double edge sword. In normal epithelial cells it has potent tumor suppressor activity by inducing cytostatic changes, differentiation, or apoptotic cell death. In contrast, in premalignant or initiated cells, TGF-β acts as a tumor promotor due to its ability to induce changes in transcriptional activities that reprogram epithelial cells into mesenchymal-like cells enhancing migration, invasion, and survival processes [313]. TGF-β also plays an active role in remodeling the tumor microenvironment, increasing angiogenesis, activating fibroblasts, and suppressing immune surveillance [255]. Although the TGF-β switch from a tumor suppressor to prometastatic factor during disease progression is well documented, the molecular mechanisms governing its function as tumor suppressor or tumor promoter remain unclear.

So far, three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) have been identified in mammals; these molecules share about 97% homology [270, 338]. The TGF-β isoforms are secreted as inactive latent precursor molecules; dimers composed of the latent associated protein (LAP) and the immature TGF-β polypeptide that require activation to initiate signal transduction [325].

TGF-β signaling is initiated by ligand binding to its specific transmembrane serine/threonine kinase receptor TGF-β type II receptor (TβRII). When TGF-β binds to TβRII it induces dimerization with TGF-β type I receptor (TβRI) [306]. In this complex, receptor TβRII phosphorylates TβRI at the GS region [310, 372, 374]. Phosphorylated TβRI specifically recognizes and phosphorylates intracellular substrates that initiate intracellular signaling events. The canonical signal messengers activated by TβRs engagement are a family of transcription factors called SMAD proteins. SMADs are classified in three subfamilies of proteins: receptor-regulated SMADs (R-SMADs), common partner SMADs (Co-SMADs), and inhibitory SMADs (I-SMADs) [249]. R-SMADs directly interact and become phosphorylated by TβRI. In mammals, SMAD2 and SMAD3 are TGF-β/Activin specific R-SMADs, whereas SMAD1, SMAD5, and SMAD8 are BMP-specific R-SMADs. The SMAD4

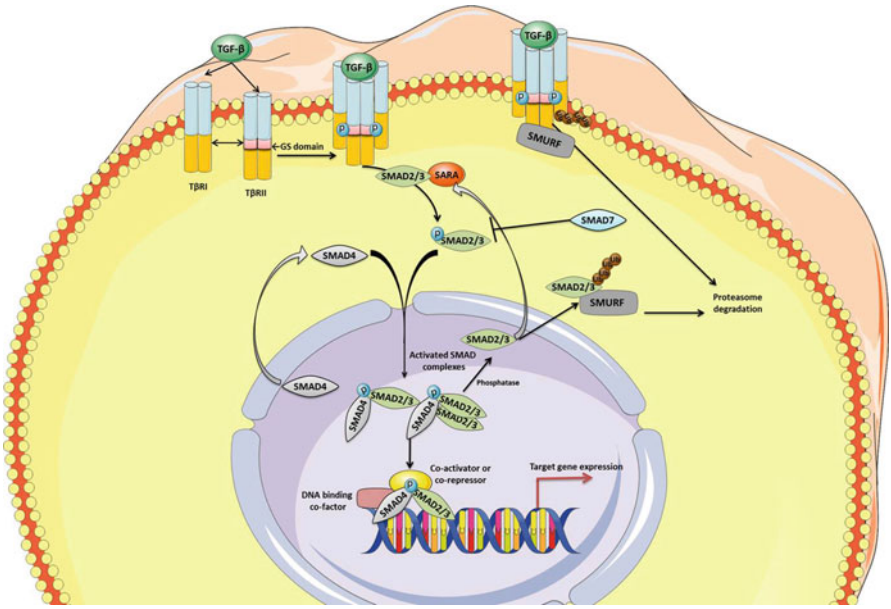

Fig. 6.3 Schematic representation of the TGF-β signaling pathway

is the only Co-SMAD known in mammals. The I-SMADs subfamily is composed of SMAD6 and SMAD7. The inhibitory function of I-SMADS is accomplished by two mechanisms. First, SMADs compete with R-SMADs for TβRI binding and; second, I-SMADS recruits SMAD ubiquitin regulatory factor E3 ubiquitin ligase (SMURF) to the activated receptor, which targets the receptor complex for proteasomal degradation. Alternatively, R-SMADs can also become ubiquitinated by SMURF and degraded by proteasomes [381].

In basal states, SMAD2 and SMAD3 can bind several proteins including SMAD-anchor for receptor activation (SARA) [363]. Such interactions retain SMAD2 and SMAD3 in the cytoplasm. Upon receptor activation, SARA brings SMADs to the activated TGF-β receptor complex where SMADS are phosphorylated by TβRII serine/threonine kinase activity. Such phosphorylation decreases the affinity of R-SMADs for SARA. Once released from SARA, SMAD2 and/or SMAD3 interact with SMAD4 assembling dimers or trimers of R-SMAD proteins that translocate to the nucleus. The activated SMAD4–R-SMAD complex can bind other DNA-binding transcription factors as partners that regulate target gene recognition and transcriptional regulation. Transcriptomic-profiling analyses have revealed that TGF-β addition leads to the rapid activation or repression of several hundred genes in a given cell type [313, 352]. Furthermore, depending on the nature of the partner, the SMAD complex will interact with transcriptional coactivators or corepressors [260]. Finally, different signals induce expression of I-SMADs which in cooperation with various E3 ligases inhibit TGF-β signaling [290, 291] (Fig. 6.3).

The identification of SMADs proteins enhanced the field of TGF-β signaling, but it also induced a dilemma in terms of reconciling the diverse functions of TGF-β family within the simplicity of the SMAD signaling node. At the present, mounting evidence has revealed that the diversity of the TGF-β signaling response is determined by the combinatorial usage of the core TGF-β pathway components with other pathways that are collectively referred to as "noncanonical" TGF-β signaling pathways. These noncanonical TGF-β pathways include various branches of MAP kinase pathways, Rho-like GTPase signaling pathways and PI3K/AKT/mTOR pathways [378].

Alterations in TGF-β Pathway in Endometrial Cancer

The mechanism of endometrial carcinogenesis is poorly understood; however, growing evidence shows that the TGF-β family members may have a role in the neoplastic transformation of human endometrium. Disruption and/or dysregulation of TGF-β signaling pathway may facilitate invasion, metastasis, and angiogenesis [246, 313].

TGF-β Isoforms

Several studies have demonstrated alterations in TGF-β isoform expression during progression from complex hyperplasia to endometrial carcinoma [247, 333, 367]. For example, it has been demonstrated that TGF-β1 acts as a paracrine factor to regulate endometrial cell proliferation [247] and changes in its expression may contribute to the neoplastic transformation of the endometrium [247]. Variations of TGF-β1 expression are not only restricted to reduced TGF-β1 mRNA levels in endometrial cancer as compared to nontumoral tissue, but differences in cell-specific expression patterns are also observed [286, 327, 330]. Particularly, a significant and progressive increase in TGF-β1 protein expression has been observed from normal proliferative endometrium to simple hyperplasia. However, no additional increase in TGF-β1 protein expression was noted with progression from complex hyperplasia to carcinoma, suggesting that dysregulation of TGF-β1 signaling is an early event in carcinogenesis [247]. The recent massive analysis of endometrial carcinoma specimens has determined that altered expression of TGF-β1 and TGF-β3 occurs in 5 % and 6 % of endometrial endometrioid carcinomas, respectively [13]. Furthermore, it has been published that TGF-β3 confers metastatic properties to endometrioid cancer cell lines by promoting cell survival and invasiveness in cell lines. Moreover, these results correlate with clinical data, which show increased TGF-β3 expression upon carcinoma progression (from stage I to stage III). TGF-β3 immunoreactivity gradually extends from epithelial compartment (in normal tissue) to the stroma (in adenocarcinoma) [367]. Additionally, it has been described that TGF-β1 is a limiting and critical factor associated with high risk of recurrence phenotype in endometrial carcinomas;

initiating tumor infiltration through the promotion of epithelial–mesenchymal transition (EMT) phenotype during myometrial invasion [303, 324].

In conclusion, dysregulation of TGF-β isoform (both at the mRNA and protein level) expression is an early event during tumorigenic transformation of the endometrium.

TGF-β Receptors

Mutation of *TβRI* (5 % of EEC) and *TβRII* (6 % of EEC) are relatively infrequent in endometrial carcinoma compared to other types of cancer [13, 326]. Data from a study analyzing *TβRI* and *TβRII* mutations in human sporadic endometrial tumors have shown that endometrial tumors contain a silent polymorphism at codon 389 in *TβRII* in 44 % of analyzed tumors samples [326]. Moreover, frame shift mutations of *TβRII* are significantly associated with microsatellite instability and closely linked with *MLH1* promoter methylation [295]. In addition, some endometrial cancers may exhibit additional changes in protein turnover and/or dysregulated endocytosis of TβRII [331]. Of note, increased protein levels of TβRII were present in endometrial cancers with myometrial invasion compared to noninvasive tumors [333].

Finally, it has been recently published that deletion of *TβRI* in mice enhances epithelial proliferation which culminates in endometrial hyperplasia in aged females. This evidence supports the role of TβRI in endometrial epithelial cell proliferation in the pathogenesis of endometrial hyperplasia [284].

Little is known about the expression pattern and regulation of the accessory TGF-β receptors (β-Glycan and CD105) in endometrial cancers. Several studies support the hypothesis that CD105 could be used as a marker for tumoral transformation of the endometrium as well as a strong predictor of reduced survival [339, 341]. Regarding β-glycan, a study suggests that downregulation of its expression is correlated with tumor differentiation. Specifically, well-differentiated tumor cells are characterized by low levels of β-Glycan staining, while poorly differentiated cells do not express β-Glycan [280].

SMAD Proteins

To date, little is known about the consequences of *SMAD* gene mutations in cancers arising from hormone-dependent tissues; moreover, the information and results published are remarkably contradictory. SMAD proteins can be considered as tumor suppressors. Inactivation and or dysregulation of SMADs expression may be a key event in tumor progression and promotion. The recent published TCGA study determined that alterations of SMADs occur in a 31 % of EC cases analyzed. Individually, the percentages of each SMAD protein alteration are as follows: 13 % of SMAD2, 7 %-SMAD3, 10 %-SMAD4, 7 %-SMAD5, and 10 % of SMAD7 [13].

Moreover, loss of heterozygosity (LOH) at the 18q21 locus, where the *SMAD2, SMAD4,* and *SMAD7* genes are located, is frequent in endometrial cancers and in most cases is correlated with a deletion at the 18q21 region where *SMAD4* is located [361]. In contrast, another study suggests that, although the LOH in this region is very frequent in EC, inactivation of *SMAD4* gene is relatively rare [307]. The expression of SMAD4 is detectable in hyperplasia, primary and metastatic EC, even though progressive reduction of its protein expression was noted with increasing tumor grade [307]. Infiltrating ECs have been characterized by significant lower mRNA levels of SMAD2 and SMAD4 in comparison to noninfiltrating ECs. Additionally, a decrease of SMAD4 expression was noted in poorly differentiated endometrial cancers compared to well differentiated; although SMAD4 levels were significantly higher in the cytoplasmic fractions [333]. Other authors have described changes in SMADs intracellular distribution during endometrial tumor progression, supporting the hypothesis that the intracellular distribution of SMADs is critical for local invasiveness of endometrial carcinogenesis [332].

So far, 10 % of EC have alterations in SMAD7 expression, with increased expression being the most frequent alteration [13]. Despite these alterations, SMAD7 expression levels do not correlate with tumor differentiation [273]. Reduced or absent phosphorylation of SMAD2/3 has been correlated with high levels of SMAD7 expression [327], suggesting that attenuation of TGF-β signaling by overexpression of SMAD7 may be important for endometrial carcinogenesis.

TGF-β Signaling Inhibitors

The genetic and preclinical studies support targeting TGF-β signaling as therapeutic strategies for combating EC. To date, there are four major TGF-β signaling antagonist approaches under development. They are as follows: (1) ligand traps: which serve as a sink for the excess of TGF-β produced by tumor cells during cancer progression. Ligand traps include antiligand neutralizing and soluble decoy receptor proteins [267]. (2) Antisense oligonucleotides which are also used to reduce the bioavailability of active TGF-β ligands in the local tumor microenvironment [337]. (3) Small molecules receptor kinase inhibitors that act via ATP-competitive inhibition of the kinase catalytic activity of the receptor [323] and finally (4) peptide aptamers which are small peptide molecules, containing a target binding domain where TGF-β signaling molecules, such as SMADs, can bind and interfere with its functions [349]. For each of these approaches, several drugs have been developed and are either in nonclinical or in early stages of clinical investigation in various cancer types. However, regarding endometrial cancer, very little has been done and further detailed studies should be performed. Nonetheless, taking into account all the observations, the potential utility of TGF-β signaling antagonist agents could be a potential novel treatment for certain advanced endometrial carcinomas.

Published Results on Endometrial Cancer Clinical Trials

Over the past 20 years, options for patients with recurrent endometrial cancer have been chemotherapy, hormonal therapy, and radiation, but none of these options have showed greatly improved mortality rates.

As described, endometrial carcinomas exhibit distinct molecular alterations that represent potential druggable targets. In this section, we will summarize some of the inhibitors used in published EC clinical trials.

PI3K/AKT/mTOR pathway is the most frequently altered signaling pathway in EC, through the high incidence of *PTEN* mutation. For that reason, increased PI3K/AKT/mTOR pathway activity has led to the development of several mTOR inhibitors such as Temsirolimus, Ridaforolimus, and Everolimus.

A phase II trial of Temsirolimus showed a 14 % response rate in chemotherapy-naive patients and a 4 % response rate in pretreated endometrial cancer [152]. Ridaforolimus, a selective mTOR inhibitor, was also evaluated in a phase II trial among 45 patients with advanced or recurrent endometrial cancer. In this study, 28 % of the patients had a clinical response, defined as complete response, partial response, or stable disease, for at least 16 weeks [153].

A third phase II trial evaluated Everolimus efficacy among 44 patients with advanced or recurrent endometrial cancer refractory to one or two previous chemotherapy regimens. The 6-month nonprogressive disease rate was 36 %, and four patients (9 %) showed partial response [154].

Given these modest response rates with single mTOR inhibitors, new drugs and combinations are being explored. Many studies have pointed out that aberrant PI3K/AKT/mTOR signaling pathway is associated with resistance to endocrine therapies in breast cancer. In this regard, a phase III showed that mTOR inhibitors may reverse resistance to endocrine therapy in breast cancer [252]. A recent phase II trial done with 38 patients with recurrent endometrial carcinoma treated with Everolimus plus Letrozole achieved a response rate of 32 % with 9 complete responses, and 2 partial responses (none with serous histology) [161]. Higher response rates were seen in patients who previously were treated with metformin. The clinical activity of metformin is now being tested in several clinical trials, including studies with endometrial cancer. At present, an open-label phase II activity trial evaluating Everolimus, Letrozole, and Metformin in endometrial cancer patients is ongoing.

Another attractive target in EC is EGFR, which is frequently overexpressed in endometrial carcinogenesis. EGFR inhibitors, such as Gefitinib and Erlotinib (Tyrosine kinase inhibitors) have been investigated in endometrial cancer patients with modest response rates of 3.4 % and 12.5 %, respectively [151]. Moreover, clinical response does not correlate with molecular features including EGFR expression by immunohistochemistry, *EGFR* mutations, or gene amplification.

Trastuzumab and Lapatinib are human EGFR type 2 (ErbB2)-related inhibitors. Trastuzumab is a monoclonal antibody against the extracellular domain of ErbB2. Lapatinib acts as a dual inhibitor of both EGFR and ErbB2 tyrosine kinase receptors. A phase II trial using Trastuzumab as a single agent in advanced or recurrent

endometrial cancer did not demonstrate any activity in endometrial cancers overexpressing ErbB2 [148]. However, several case reports have demonstrated that Trastuzumab may be useful in uterine serous adenocarcinomas (USC), because it has been described that *ErbB2* is overexpressed in 18–62 % of USC. Moreover, a phase II ongoing study is evaluating whether the addition of Trastuzumab to Paclitaxel and Carboplatin chemotherapy improves progression-free survival in EC stages III–IV and recurrent USC patients overexpressing ErbB2/Neu.

Other signaling pathway inhibitors used in EC clinical trials are selective angiogenesis inhibitors. Bevacizumab is a recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting vascular endothelial growth factor A (VEGF-A). A phase II trial using Bevacizumab in patients with recurrent or persistent endometrial cancer after one or two prior chemotherapy regimens showed a response rate of 13.5 % (one of 53 patients showed a complete response) [156]. Given the promising results seen in other gynecological malignancies, a three-arm randomized phase II trial is being developed in patients with advanced or recurrent disease. This study is evaluating standard paclitaxel/carboplatin chemotherapy in combination with either Bevacizumab, or Temsirolimus, while a third arm will evaluate Ixabepilone/Carboplatin and Bevacizumab.

In addition to monoclonal antibodies, there are several small molecule inhibitors, which have been designed to target tyrosine kinase receptors, such as Sunitinib or Sorafenib. These inhibitors have exhibited modest activity, with response rates of 15 % and 5 %, respectively [158, 159].

Brivanib, an oral, multitargeted tyrosine kinase inhibitor has also been tested as a single agent in a phase II trial in recurrent or persistent endometrial cancer, showing a response rate of 18 %, including one complete response and seven partial responses [157].

The complexity and heterogeneity of EC may explain why different target-specific inhibitors used effectively during a period can become insufficient after repeated rounds of treatment. Single drug agents can result in resistance to the chemotherapy or development of multidrug resistance.

Combined therapies overcome side effects associated with high doses of single-agent drugs, enabling a low dose of each compound while accessing context-specific multitarget mechanisms. Although preclinical trial data have revealed rational therapeutic approaches for combined therapy, further clinical validation should be performed.

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Part III
Mouse Models

Chapter 7
LKB1 as a Tumor Suppressor in Uterine Cancer: Mouse Models and Translational Studies

Christopher G. Peña and Diego H. Castrillón

Abstract The *LKB1* tumor suppressor was identified in 1998 as the gene mutated in the Peutz–Jeghers Syndrome (PJS), a hereditary cancer predisposition characterized by gastrointestinal polyposis and a high incidence of cancers, particularly carcinomas, at a variety of anatomic sites including the gastrointestinal tract, lung, and female reproductive tract. Women with PJS have a high incidence of carcinomas of the uterine corpus (endometrium) and cervix. The *LKB1* gene is also somatically mutated in human cancers arising at these sites. Work in mouse models has highlighted the potency of LKB1 as an endometrial tumor suppressor and its distinctive roles in driving invasive and metastatic growth. These in vivo models represent tractable experimental systems for the discovery of underlying biological principles and molecular processes regulated by LKB1 in the context of tumorigenesis and also serve as useful preclinical model systems for experimental therapeutics. Here we review LKB1's known roles in mTOR signaling, metabolism, and cell polarity, with an emphasis on human pathology and mouse models relevant to uterine carcinogenesis, including cancers of the uterine corpus and cervix.

Keywords LKB1 • STK11 • Endometrial cancer • Uterine cancer • Genetically engineered mouse models • MTOR • AMPK • Therapeutics

Introduction

In humans, the *LKB1* (*Liver Kinase B1*) gene, a.k.a. *STK11* (*Serine Threonine Kinase 11*), is located on chromosome 19p13.3 and encodes a serine/threonine kinase with important roles in human disease, particularly cancer [1]. The *LKB1*

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gene contains 9 coding exons [2], resulting in a 433 amino acid intracellular kinase (48 kDa) [3] that regulates diverse aspects of cellular physiology including metabolism, growth and proliferation, and cellular polarity, among other functions. Ubiquitous expression of LKB1 in adult tissues [4] and its conservation from fruit flies to mammals [5], together with many functional investigations into its biological roles in these diverse organisms, have established the universality of many of these essential cellular functions. In mammals, germline (hereditary) or somatic (acquired) mutations in *LKB1* provoke a variety of tumors.

LKB1 was originally identified as the gene responsible for the Peutz–Jeghers Syndrome (PJS), an autosomal dominant condition characterized by polyposis of the gastrointestinal tract, mucocutaneous hyperpigmentation (i.e., perioral), and a dramatically increased risk for cancers throughout the body [6]. These individuals are born with one mutant (loss of function) and one normal allele of *LKB1*. Subsequent investigations confirmed that LKB1 is a classic tumor suppressor, where biallelic inactivation is required to give rise to the most potent growth and tumor-promoting phenotypes. However, considerable evidence points to the fact that LKB1 can function as a haploinsufficient tumor suppressor. For example, many intestinal polyps do not undergo loss or mutation of the second allele [7–9]. Furthermore, downregulation of LKB1 by diverse epigenetic or posttranslational mechanisms has been strongly implicated in malignant transformation of many organs including the breast, colon, lung, skin, and cervix [10–15].

Here, we review a growing literature implicating LKB1 in the normal physiology and malignant transformation of the uterus. A variety of translational studies employing human material, together with genetically engineered mouse models, have studied LKB1 in endometrial carcinogenesis. In addition, LKB1 participates in related malignancies of the lower female reproductive tract including the cervix, oviduct, and ovary, arguing that LKB1 functions as a tumor suppressor throughout the Müllerian tract and its derivatives. Loss of LKB1 protein is observed in ~20 % of primary endometrial cancers, and mouse models have revealed a uniquely potent role of LKB1 as an endometrial tumor suppressor [16–19]. Loss of LKB1 in endometrial adenocarcinoma mouse models is associated with striking invasion and rapid disease progression and spread, leading to early death [17, 18, 20, 21]. To better frame these results, basic LKB1 biology and genetics will be discussed, highlighting diverse mechanisms of LKB1 loss and the diverse biological and biochemical pathways impacted by LKB1 inactivation. Genetically engineered mouse models (GEMMs) based on conditional inactivation of *LKB1* in the uterus, oviduct, and ovary will be reviewed, as well as their potential uses in discovering novel modes of LKB1 action and as preclinical platforms to test new therapeutic approaches.

Tumor Spectrum and Reproductive Tract Malignancies Associated with PJS

Individuals with PJS are at increased risk for cancers throughout the body. Interestingly, the vast majority of these cancers are of epithelial origin (i.e., carcinomas), and the incidence of nonepithelial malignancies (sarcomas and lymphomas)

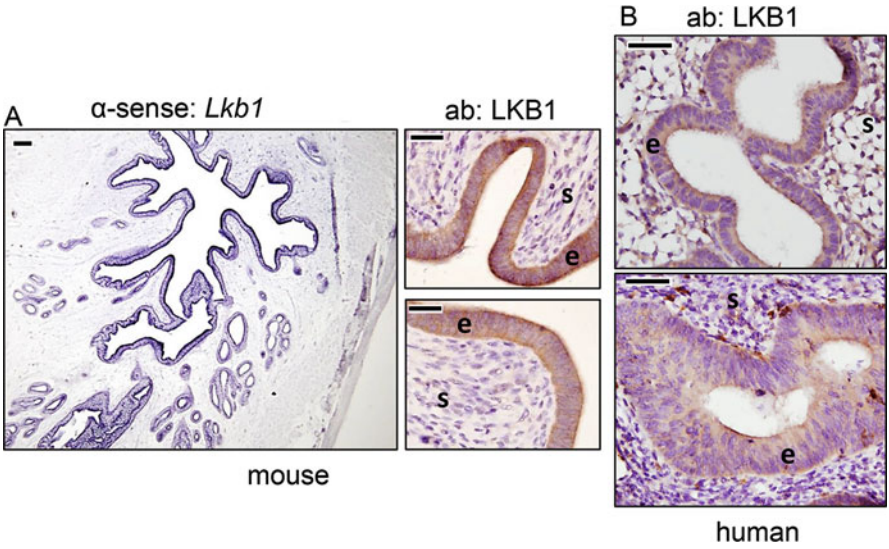

Fig. 7.1 LKB1 is expressed in mammalian endometrial epithelium. (**a**) In situ RNA hybridization with an LKB1 α-sense probe (*left*) and immunohistochemistry (*right*) of murine endometrium reveals high LKB1 expression in epithelial cells compared to stroma. (**b**) IHC staining for LKB1 shows high expression in human endometrial epithelium. S denotes stroma, e denotes epithelium. Scale bars = 50 μm

does not appear to be significantly elevated. Although the biological basis for this predilection for carcinomas is likely multifaceted, it is notable that LKB1 is most highly expressed in the epithelial compartment of diverse organs, suggesting a more potent functional role in the epithelial vs. mesenchymal compartments of diverse organs. For example, LKB1 is more highly expressed in endometrial epithelium than in other uterine compartments (Fig. 7.1). However, this notion is undoubtedly an oversimplification, as LKB1 does have definitive functional roles in nonepithelial cell types, e.g., in hematopoiesis [22–24] and in stroma [25]. These observations and the subsequent identification of spontaneous (i.e., noninherited) *LKB1* mutations in diverse carcinomas (but not sarcomas or lymphomas) demonstrate that LKB1 is remarkably specific as an epithelial tumor suppressor.

The most frequent sites of malignancy in PJS are the gastrointestinal tract (including the esophagus, stomach, pancreas, and intestine), lung, breast, and the lower female reproductive tract [10, 26, 27], sites where spontaneous *LKB1* mutations have also been described in tumors. Unfortunately, studies documenting tumor spectra in women with PJS have tended to catalog gynecologic (i.e., lower female reproductive tract) malignancies together, making it difficult to make specific statements about the relative incidence of cervical vs. endometrial vs. ovarian cancer in these patients. However, the risk of all three of these lower reproductive tract cancers is clearly elevated in PJS. For example, a multicenter study reported a relative cancer risk of 55.6 for "cervix" (95 % confidence interval 17.7–134.0) and 27.7 (95 % confidence interval 11.3–57.6) for "gynecologic cancers" [27]. The cumulative

risk from age 15 to 64 of uterine, ovarian, and cervical cancer in women with PJS has been estimated at 9 %, 21 %, and 10 %, respectively (with a cumulative cancer risk at all sites throughout the body of 93 %) [11].

PJS is associated with two highly distinctive neoplasms of the female reproductive tract, including the ovary and the uterine cervix. Minimal deviation adenocarcinoma (MDA) of the endocervix (a.k.a. adenoma malignum) is an extremely well-differentiated variant of endocervical adenocarcinoma strongly associated with PJS, although MDAs exhibiting this histology can also occur sporadically. Paradoxically, although MDAs can be difficult to diagnose histopathologically due to their resemblance to normal endocervical glands and overall well-differentiated appearance (Fig. 7.2), these tumors are very aggressive and locally invasive [28]. These observations were an early indication (even before the gene was cloned) that

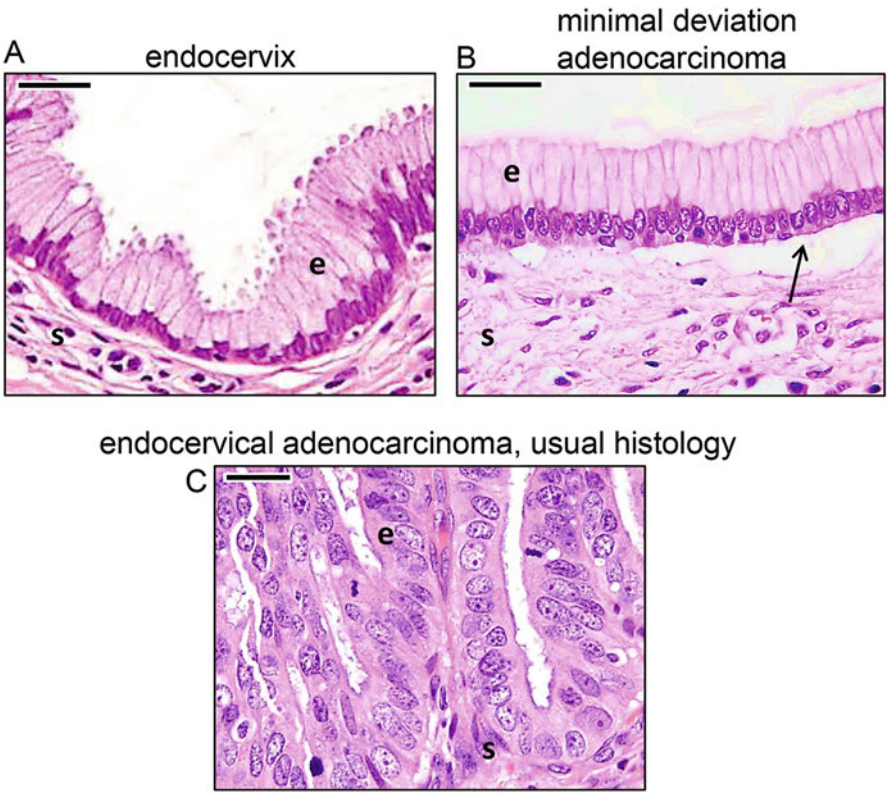

Fig. 7.2 Peutz–Jeghers Syndrome and association with well-differentiated endocervical adenocarcinomas. (**a**) Normal endocervix. (**b**) Minimal deviation adenocarcinoma (MDA) (a.k.a. adenoma malignum), an extremely well-differentiated endocervical adenocarcinoma that closely resembles normal endocervix. Note the extremely well-polarized appearance of the epithelium and retraction of epithelium from underlying stroma, a histologic clue for the diagnosis of MDA (*arrow*). (**c**) Well-differentiated endocervical adenocarcinoma, usual histology. S denotes stroma, e denotes epithelium. All images are from H&E stained sections. Scale bars = 50 μm

the factor encoded by the PJS locus had unique biological functions in promoting invasion, and might thus be distinct from classical tumor suppressors (e.g., *TP53*, *RB*) that act principally by regulating cell cycle progression and cellular survival. MDAs (either spontaneous or in PJS) are HPV-negative and do not arise from pre-existing dysplastic lesions (high-grade squamous intraepithelial lesions/HSILs), distinguishing them from the vast majority of cervical cancers [29, 30]. *LKB1* mutations (deletions or point mutations) occur in about 20 % of primary (HPV-positive) cervical cancers across histologic subtypes including endocervical adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, and have also been documented in (HPV-negative) MDAs [31]. LKB1 loss thus almost certainly synergizes with HPV to convert otherwise noninvasive SILs into invasive cancer. However, the true incidence of LKB1 mutations in spontaneous MDAs is unknown, in part because of the difficulties of detecting the wide range of LKB1 mutations and deletions that can result in functional inactivation of the locus.

Lobular endocervical glandular hyperplasia (LEGH), originally described as a pseudoneoplastic benign lesion of the endocervix, is a histologically distinctive lesion characterized by a striking lobular proliferation of small endocervical glands in a pattern that can mimic MDA, but with no evidence of the epithelial atypia, stromal reaction, or the deep invasion that characterizes MDA. LEGH is usually an incidental microscopic finding, but sometimes can form a discrete mass. More recently however, the presence of LEGH has been reported in women with PJS, sometimes concurrently with MDA [32, 33]. While MDA is the more common lesion in the context of PJS, MDA and LEGH remain histologically distinct [34]. The concurrence of LEGH and MDA in some women, together with the identification of microscopic foci of cytologic atypia in some cases of LEGH, suggests that LEGH can serve as a precursor lesion for MDA [35, 36]. Concordantly, a recent study identified *LKB1* mutations in 2/19 cases of LEGH. Molecular analyses of additional cases of LEGH perhaps combined with LKB1 immunohistochemistry [37] would likely shed further light on the relationship between MDA and LEGH. It is also interesting to speculate that rare variants of endometrial adenocarcinoma associated with highly infiltrative, "MDA-like" patterns of invasion and infiltration, such as "endometrioid adenocarcinoma with a minimal deviation invasive pattern" [38], or "diffusely infiltrative endometrial adenocarcinomas" [39] might be specifically associated with LKB1 inactivation or downregulation.

In the ovary, an unusual (and again, histologically distinctive) variant of granulosa cell tumor known as a "sex cord tumor with annular tubules" (SCTAT) is strongly associated with PJS. Although the majority of SCTATs are sporadic, occurring in girls or women not known to have PJS, they are a common finding in women with PJS. Reflecting their granulosa cell origin, SCTATs are often hormonally active and can be associated with clinical signs of hyperestrinism, including postmenopausal bleeding and endometrial hyperplasia. Like most granulosa cell tumors, SCTATs are often confined to the ovary at the time of diagnosis, but they sometimes metastasize and can be fatal [40]. The presence of bilateral SCTATs is considered to be virtually pathognomonic for PJS, and several cases have been described of women with PJS presenting with simultaneous bilateral SCTATs and MDA [41].

No mouse models of human SCTAT or cervical MDA have been described, although conditional inactivation of *LKB1* in mouse endometrium (described in detail later) yields extremely well-differentiated endometrial cancers that are paradoxically invasive and biologically aggressive, thus sharing some salient properties with human MDA and endometrial adenocarcinomas with MDA-like infiltration patterns [17, 18].

While there are no published case series describing the spectrum of endometrial cancers in PJS women, there is no suggestion in the literature—unlike the PJS tumors of the cervix—that such tumors are histologically distinctive or unique. Similarly, the clinical and histopathologic characteristics and histologic range of surface epithelial tumors of the ovary in PJS have not been described in the literature. However, both serous cystadenomas and ovarian carcinomas have been reported, which, in the absence of reports to the contrary, may be presumed to exhibit classic serous histology [27]. However, it would clearly be of interest to have more granular and extensive information on the incidence, histological subtypes, and clinical behavior of the diverse upper reproductive tract malignancies in women with PJS.

Some earlier studies raised the specter of a second PJS locus [42, 43], but recent studies have suggested that virtually all cases of PJS are attributable to mutations in *LKB1*. The failure to detect *LKB1* mutations in some patients in the earlier studies now appears to reflect the diverse and highly divergent types of mutations that can functionally inactivate *LKB1*, leading to false negatives. In addition to point mutations (single amino acid substitutions, nonsense/frameshift mutations), the locus is highly prone to deletions, which can be large (up to 100 kb or more and extend to neighboring loci), or small and intragenic. Such intragenic deletions can range from tens of kilobases to small subexonic deletions of just a few bases and can be readily missed by standard targeted gene resequencing or whole-exome techniques. For example, HeLa, which was long known to be LKB1-null at the protein level, harbors a homozygous 25 kb deletion within the 5′ end of the locus removing the promoter and the first three exons, and the mutation was shown to have occurred in vivo (i.e., it was not an in vitro culture artifact) [31]. Thus, careful analysis and specialized techniques such as multiplex-ligation probe amplification (MLPA) may be needed to systematically identify inherited *LKB1* mutations [44, 45]. This has also made it challenging to identify spontaneous mutations in human tumors, since no one test (whole-genome sequencing included) reliably detects *LKB1* mutations, likely explaining the tendency for most studies to underestimate LKB1 mutation frequency. Finally, it is also worth noting that although dozens of distinct *LKB1* mutations have been identified in individuals with PJS, no convincing mutation–phenotype correlations have been established with respect to tumor incidence, tumor spectrum, or severity of any aspect of the syndrome [3, 6, 10, 27, 45–47]. This is consistent with the notion that these mutations are largely functionally equivalent, leading to loss of LKB1 function. So although it could reasonably be expected that weak, hypomorphic *LKB1* mutations would lead to "*formes frustes*" of PJS, such mutations or clinical variants of PJS have not yet been described.

LKB1 Structure, Regulation, and Binding Proteins in Mammalian Cells

The LKB1 protein (433 a.a.) consists of a central catalytic protein kinase domain flanked by N- and C-terminal regulatory domains [48]. The great majority of inactivating *LKB1* mutations occur within the kinase domain [49]. Phosphorylation of LKB1 in the regulatory domains can occur at 11 total sites, of which Thr185, Thr189, Thr336, and Ser404 are direct targets of LKB1 itself (autophosphorylation). Phosphorylation of these sites does not affect kinase activity or subcellular localization in vitro, but serves as one indicator of catalytically active LKB1, whereas other sites (Ser31, Ser307, Ser325, Thr366, Ser399, Ser 428, and Ser431) are phosphorylated by upstream kinases (cAMP-dependent protein kinase a.k.a. protein kinase C, ataxia telangiectasia mutated kinase, and DNA-dependent protein kinase) and influence LKB1 cytoplasmic translocation as well as LKB1-dependent growth suppression (Fig. 7.3) [48, 50–55].

LKB1 kinase activity is governed by the heterotrimeric complex formed by the association of LKB1 with two proteins, sterile-20-related adaptor (STRAD) and mouse protein 25 (MO25). MO25 serves as a scaffolding protein that binds to the C-terminus of STRAD, enhancing its binding to LKB1. STRAD, a pseudokinase,

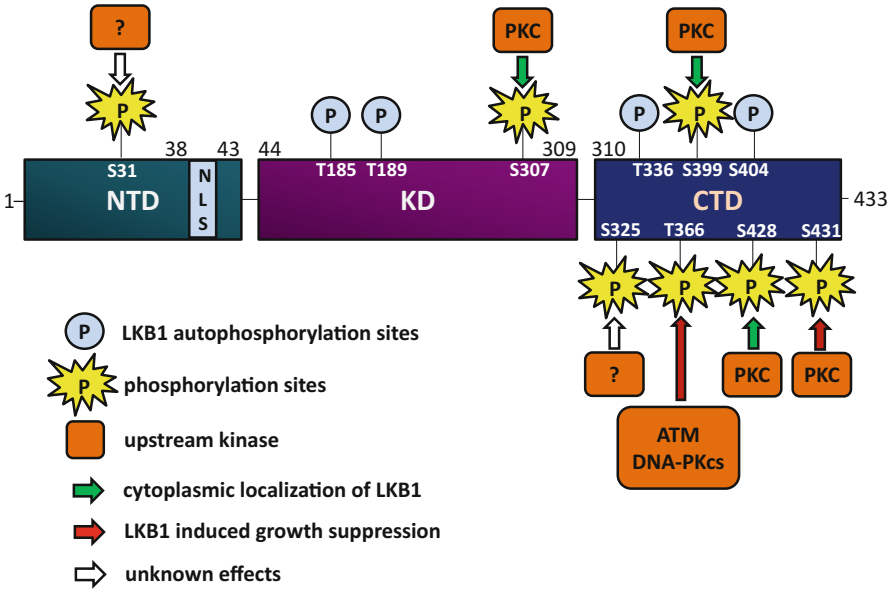

Fig. 7.3 Map of human LKB1 amino acid sequence and known phosphorylation sites. LKB1 localization and ability to induce growth suppression are modulated in part by phosphorylation of key amino acids. Shown are known phosphorylated residues, upstream kinases, and effects of phosphorylation status. *NTD* N-terminal domain, *KD* kinase domain, *CTD* C terminal domain, *NLS* nuclear localization sequence, ?=unknown kinase(s), *PKC* protein kinase c, *ATM/DNA-PKcs* ataxia telangiectasia mutated kinase/DNA-dependent protein kinase

subsequently promotes the active confirmation of LKB1 [56, 57]. In vitro models have shown the interaction of these two proteins with LKB1 is critical for constitutive kinase activity [58, 59]. The STRAD/MO25 complex is equally essential for translocating LKB1 from the nucleus to the cytoplasm and cell membrane, where it performs the majority of its functions [59]. Paradoxically (given this obligate functional interrelationship), germline mutations in neither *STRAD* nor *MO25* have been identified in PJS, nor have acquired *STRAD* or *MO25* mutations been identified in sporadic tumors [49, 60, 61].

Whereas phosphorylation can affect LKB1 activity and localization, ubiquitination has been implicated in the stabilization of LKB1. Pull-down experiments have shown an association of the molecular chaperones heat-shock protein 90 (HSP90) and cell-division cycle 37 (CDC37) with the kinase domain of LKB1. Pharmacological inhibition of these molecular chaperones resulted in ubiquitination and degradation of LKB1 in the proteasome [62], suggesting their function is to stabilize LKB1 during times of cellular stress. Paradoxically, this interaction was also shown to reduce LKB1 kinase activity [63]. As LKB1 plays a central role in regulating cell behavior during metabolic stress (described later in more detail), such mechanisms likely serve to preserve LKB1 during times of cellular stress when LKB1 activity is critically needed for cellular metabolic adaptation. Also, these and other posttranslational mechanisms regulating LKB1 activity and stability represent viable mechanisms for functional LKB1 inactivation in tumors in the absence of mutations.

LKB1 Substrates: Identification of AMPK as the Canonical LKB1 Target and Subsequent Identification of AMPK-Related Kinase Family Members as LKB1 Targets

AMP-activated protein kinase (AMPK) is a sensor of energy charge that is activated by the rising AMP that accompanies a fall in the ATP:ADP ratio. Once activated by a drop in ATP levels, AMPK switches on the uptake of glucose and fatty acids and oxidative metabolism to generate ATP, while switching off biochemical pathways that utilize ATP, thereby conserving energy [64]. Activated AMPK inhibits anabolic pathways such as fatty acid and cholesterol synthesis through phosphorylation of the metabolic enzymes Acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (HMGR) [65]. AMPK also decreases ATP-consuming processes such as protein synthesis and cell growth [66] by regulation of the mTOR pathway. Activated AMPK (pAMPKThr172) phosphorylates the tuberous sclerosis tumor suppressor complex 1 (TSC1) [67] and the raptor proteins [68]; the former inhibits mTOR signaling through the GTPAse Rheb [69, 70] and the latter, when phosphorylated, inhibits mTOR by recruitment of the 14-3-3 adaptor protein to mTOR [68]. The net result of either process is the inability of mTOR to activate key proteins, ribosomal S6 (S6) and eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), which are involved in the translation of mitogen stimulated mRNAs responsible for cell cycle initiation and proliferation (Fig. 7.4) [67, 71].

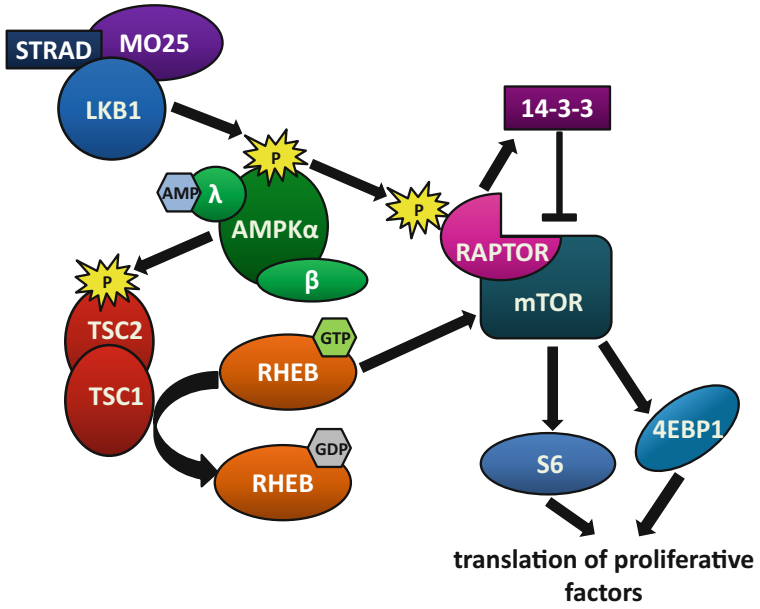

Fig. 7.4 LKB1 regulates the mTOR pathway. Under metabolic stress (low ATP, high AMP levels), LKB1 phosphorylates AMPK, which inhibits downstream translation of proliferative factors by inhibiting the mTOR pathway. This occurs by either phosphorylation of the TSC complex and inactivation of the GTPase Rheb or by direct phosphorylation of Raptor, an mTOR binding partner that inhibits mTOR through the recruitment of 14-3-3 adaptor proteins

AMPK is a heterotrimer consisting of a catalytic subunit (AMPKα) and two regulatory subunits (AMPKβ and AMPKγ). The β subunit is a scaffolding protein on which the AMPK complex assembles, whereas the γ subunit facilitates binding to AMP [72]. AMPK is fully active when AMP binds the AMPK complex at the cystathionine-β-synthase (CBS) domain located on AMPKγ, which in turn stimulates the phosphorylation of Thr172 in the activation "T" loop of the catalytic subunits AMPKα1 and 2 [73]. AMP binding to the γ subunit induces a conformational change that can inhibit dephosphorylation of Thr172, thus keeping pAMPK-Thr172 in its active conformation.

AMPK was known to be activated by an upstream kinase that phosphorylated AMPK at residue Thr172 within the activation loop of the kinase domain—but the identity of this kinase was initially unclear. The identification of LKB1 as this critical kinase activator of AMPK in mammalian cells began with studies in the yeast *Saccharomyces cerevisiae*. The protein kinases elongated morphology-1 (Elm1), snf-1 activating kinase-1 (Sak1), and target of Sbf-3 (Tos3) were identified by copurification with the AMPK homolog sucrose nonfermenting-1 (Snf-1) [74, 75]. Genetic knockout of these proteins resulted in absent phosphorylation at Snf-1's threonine activation loop, significantly reducing Snf1 activity [76]. Purified Tos3 protein phosphorylated human AMPK on Thr172. Tos3's shared sequence similarity with LKB1 led to testing and confirmation of direct LKB1-mediated AMPK phosphorylation in various metazoan and mammalian models [76–78].

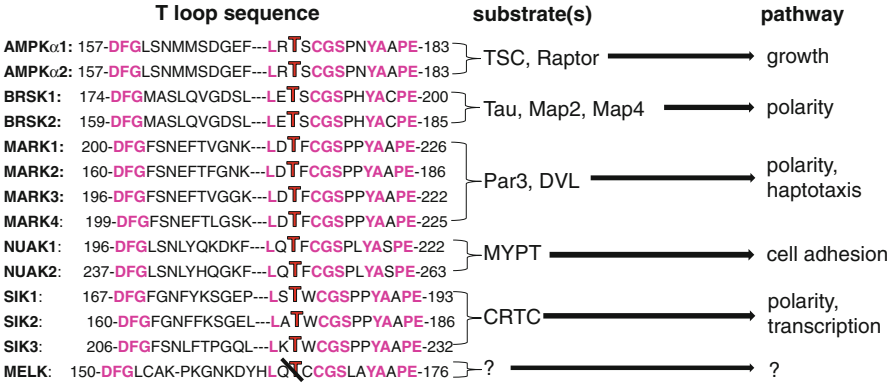

Fig. 7.5 LKB1 phosphorylation of AMPK family members occurs at conserved threonine residues. Amino acid sequences of the T-Loop activation domain in all 14 AMPK family members are shown, including their downstream substrates and biological pathways affected. Conserved residues are shown in *pink*. The threonine residue in the T-loop is indicated in red. Except for MELK, LKB1 phosphorylates T residues in all AMPK family members

Twelve other kinase homologs (known as the AMPK-related kinases) are homologous and closely related to AMPK. These kinases include NUAK1, NUAK2, BRSK1, BRSK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4, and MELK. The threonine and surrounding residues within the activation loop of AMPK were shown to be evolutionarily conserved in AMPKα2 as well as the 12 other AMPK-related kinases [79] in humans, suggesting that all AMPK-related kinases were likely substrates for LKB1 phosphorylation (Fig. 7.5). Direct phosphorylation of the AMPK-related kinases at their threonine activation loop by LKB1 greatly enhanced the activity of each AMPK-related kinase in kinase assays with the exception of MELK, thus confirming that most are true LKB1 substrates that require LKB1 for full activity. In HeLa cells, which are deficient for LKB1 due to the aforementioned 25 kb homozygous intragenic deletion [31], the activity of AMPK family members was restored by expressing wild-type *LKB1*, thus showing in vivo regulation of the AMPK-related kinases by LKB1. Thus, LKB1 functions as the master upstream protein kinase regulating not only AMPK but the entire family of 13 AMPK-related kinases [79].

Concordantly, while some of LKB1's biological effects are mediated by AMPK, a growing body of evidence has implicated other AMPK-related kinases as physiologically important effectors of discrete LKB1 biological functions. For example, LKB1 regulates epithelial cell polarity through the MARK kinases, and axon branching and cell adhesion via the NUAK kinases [80, 81]. LKB1 also controls distinct forms of cell motility—notably cell migration along extracellular matrix (haptotaxis)—through the MARK kinases [82]. Thus, LKB1 controls cellular physiology through a combination of AMPK-dependent and -independent pathways, and a major focus of current investigations into LKB1's role as a tumor suppressor rest on the delineation of the relative contributions of the AMPK-related kinases to the specific biological actions under the direct control of LKB1 (Fig. 7.5).

Regulation of Cell Polarity by LKB1

Aside from metabolism, LKB1 plays a major part in spatial organization of subcellular components, i.e., cell polarity. The discovery of a link between LKB1 and polarity was first recognized through studies of *Par-4*, the *C. elegans LKB1* homolog. *Par-4*, when inactivated by missense mutation or RNA interference, resulted in the failure of asymmetric cell divisions necessary for the development of the anterior and posterior axis in embryos [83]. In *Drosophila*, a genetic screen uncovered *LKB1* as a facilitator of anterior and posterior oocyte development. When phosphorylated by upstream kinases, *Drosophila* LKB1 also mediated polarization of epithelial cells and the microtubule cytoskeleton [84]. In mice, LKB1 was also implicated in polarization of oocytes. LKB1 protein is asymmetrically located to the animal pole of the mouse oocyte and associated with microtubules of metaphase I and II meiotic spindles [85].

AMPK itself has essential roles in the establishment of epithelial cell polarity. For example, in Madin–Darby canine kidney (MDCK) cells, LKB1 phosphorylation of AMPK was critical in the formation of epithelial tight junctions during energy stress. In MDCK cells, expression of AMPK with dominant negative mutations led to inhibition of tight junction assembly that could be rescued only through mTOR inhibition [86, 87]. The LKB1-AMPK-mTOR pathway is also required Sertoli cell polarity and tight junction formation in mouse testes [88], which may be related to the abnormal testicular phenotypes including Sertoli cell tumors that have been observed in men and boys with PJS. In mouse neurons, LKB1 phosphorylates the BRSK (SAD) kinases, resulting in activation of microtubule-associated proteins required for dendritic/axonic polarization of neurons [89]. Studies in *Drosophila* showed LKB1-induced adherens junction formation in the eye—possibly through SIK and a second AMPK-related kinase, NUAK [90]. These diverse studies implicate LKB1 in the regulation of cell polarity through diverse but tissue-specific pathways.

LKB1, Cell Polarity, and Cancer

Loss of polarity is a characteristic of many carcinomas and is believed to facilitate cancer growth through multiple mechanisms. The disruption of the mitotic spindle can promote aneuploidy in epithelial cells [91] and accumulation of cytoskeletal components at the leading edge of these cells [92], which can trigger abnormal cell motility and invasion into surrounding tissue. Misalignment of other critical cellular factors between stem and progenitor cells during cell division can confer to the latter a more “stem-like” proliferative phenotype [93]. Lastly, disruption of epithelial and tight junctions can precipitate a migratory, mesenchymal-like phenotype, thus enhancing invasive and metastatic properties [94].

The involvement of LKB1 in the control of cellular polarity in humans was first demonstrated with intestinal cancer cell lines, highlighting that the actions of LKB1 in the establishment of polarity are relevant to human cancer. Ectopic STRAD

expression in these cells (which otherwise express low to absent levels of STRAD) activated LKB1, leading to the formation of an apical brush border via cytoskeletal rearrangement and the relocation of junctional proteins ZO-1 and P120 to their proper locations [95]. The link between STRAD, LKB1, and polarity was also observed in cultured cervical cancer cell lines, where loss of LKB1 resulted in reduced STRAD protein levels and misaligned lamellipodia and golgi [96]. However, in spite of apparent loss of polarity, these cells were unable to invade through a matrigel derived membrane.

Recently, the advent of three-dimensional culturing models has enabled researchers to take a more nuanced look at epithelial polarization while taking into account the role of extracellular matrix, basement membrane, and other stromal-related proteins. A key study utilizing 3D cultures to investigate LKB1 took advantage of mouse mammary epithelial cells (MMECS). *Adeno-Cre*-mediated *LKB1* ablation resulted in abnormal morphology and delocalization of polarity markers (i.e., apical markers like GM130 were delocalized either laterally or basally). Importantly, *LKB1* deletion led to basement membrane deterioration and tumorigenesis when coupled with oncogenic MYC [92].

Involvement of LKB1-AMPK-mTOR in Cancer

Deregulation of the LKB1-AMPK-mTOR pathway has been well documented in a variety of cancer models, albeit with different outcomes based on tissue type. *LKB1* loss deregulates cell growth and proliferation, and therefore facilitates neoplastic growth by elevating mTOR signaling. Gastrointestinal polyps from $LKB1^{+/-}$ mice show elevated signaling downstream of mTOR [97]. Deletion of *LKB1* in the liver, in addition to other metabolic defects, also inhibits AMPK activity and increases mTOR signaling [98]. In an ErbB2-mediated mammary gland tumorigenesis mouse model of breast cancer, mTOR activity was increased following genetic *LKB1* inactivation [99]. Lastly, conditional deletion of *LKB1* in endometrial epithelium (in models described in greater detail later) produced invasive tumors characterized by elevated phosphorylated ribosomal S6 [18], an effect also observed in *LKB1/PTEN* double knockout animals [21] and in animals harboring *LKB1* deletion in uterine stroma [20]. Importantly, all three of these endometrial adenocarcinoma animal models display therapeutic sensitivity to mTOR inhibitors such as rapamycin and BEZ235 (described later in more detail).

There are also instances when unchecked mTOR signaling via LKB1 loss is adverse for cells, especially when nutrient availability is low. *LKB1*-null murine embryonic fibroblasts (MEFs) display hypersensitivity to apoptosis induced by energy stress compared to *LKB1* wild-type cells [100], while $LKB1^{+/-}$ (heterozygous) MEFS are resistant to transformation in combination with oncogenes such as *HRAS* [101]. Transient knockdown of AMPK via shRNA in pancreatic cancer cell lines significantly diminishes their tolerance to glucose deprivation. Additionally, stable shRNA-AMPK pancreatic cell lines do not grow in orthotopic mouse models [102]. Although the mechanism by which LKB1 loss inhibited cell growth in these

models is not entirely understood, LKB1-AMPK phosphorylation is critical for stabilization of the cell cycle-dependent kinase inhibitor (CDKI) p27, which is critical for cell survival through autophagy induction [103]. Therefore, it is not uncommon for endogenous LKB1 to activate substrates conducive to preserving cells during harsh conditions. To induce transformation of cells, the effects of losing these "prosurvival" signals must be countered by acquired effects of hyperactive mTOR signaling.

Closer examination of downstream mTOR targets further supports this argument and reconciles this paradox of aberrant LKB1-AMPK-mTOR signaling in the context of cell growth. In the nonsmall cell lung carcinoma (NSCLC) cell line A549, which displays no LKB1 expression due to a premature stop mutation Q37X [104] and is characterized by increased mTOR signaling, produces hypoxia-inducible factor-1α (HIF-1α) under normal nutrient conditions. Upon treatment with the mTOR inhibitor rapamycin, HIF-1α levels significantly dropped. Importantly, HIF-1α transformed the metabolic profile of these cells during nutrient deprivation and enabled their survival during these conditions [105]. A separate study also implicated LKB1-AMPK in the regulation of HIF-1α in MEFs [106].

Downstream targets of mTOR-HIF-1α tied to LKB1 expression can have protumorigenic effects. For example, the matrix remodeling protein lysyl oxidase (LOX), normally downregulated by the LKB1-MTOR pathway, was highly expressed in lung epithelium upon genetic *LKB1* deletion and facilitated the migration and anchorage-independent growth of lung epithelial cells [107]. Activation of MYC and SREBP1, additional transcription factors regulated by mTOR, facilitate tumor lipogenesis, cell growth, and angiogenesis in conditions of stress [108, 109]. Taken together, these results suggest that cancer cells undergoing LKB1 loss and mTOR hyperactivity can bypass cell death and loss of survival factors if they are able to upregulate (via mTOR or other mechanisms) additional survival or tumorigenic factors that allow them to adapt to adverse conditions.

The recent identification of *MTOR* mutations in a wide array of sporadic cancers has further stressed the role of mTOR signaling in carcinogenesis. The mutations occur in the C-terminal half of mTOR and are hyperactivating (i.e., gain of function), and do not affect mTOR complex assembly, but confer varying degrees of pathway activation. Interestingly, *MTOR* activating mutations were most common in colorectal and endometrial adenocarcinomas (reportedly in 11.1 % and 10.5 % of cases, respectively) but were also common (>5 % incidence) in melanoma and lung cancers; all tumors characterized by a high incidence of *LKB1* mutations. These hyperactive MTOR mutant proteins retained their sensitivity to rapamycin, and cancer cell lines that harbored such mutations were hypersensitive to growth inhibition by rapamycin [110]. It remains to be determined if such mutations are generally predictive of clinical responses to rapalogs, but the extraordinary responses to Everolimus reported in some patients whose tumors harbored *MTOR* activating mutations suggest that this may be the case [111]. This question is of special interest in endometrial cancer, since objective responses to Temsirolimus (an mTOR inhibitor) have been documented in a significant percentage of cases of advanced endometrial cancer [112]. However, no predictive biomarkers for such responses have been identified despite intensive investigations [113, 114].

LKB1 Regulation of CREB-Dependent Transcription

In addition to its previously described functions, LKB1 has potent effects in shaping the cellular transcriptome through multiple mechanisms including direct phosphorylation of CREB-regulated transcription activators [115, 116], phosphorylation by AMPK of diverse transcriptional activators such as the FOXOs [117], and suppression of MYC [118] and WNT signaling [119]. Phosphorylation of AMPK family members by LKB1 can thus regulate gene expression independent of mTOR activity. Regulation of CREB via the CREB-transcriptional coactivator (CRTC) family has emerged through multiple studies as an important general LKB1-dependent mechanism of transcriptional regulation. The CRTCs (CRTC1, 2, and 3) were identified through high-throughput screening of cDNAs that target cAMP responsive elements in luciferase vectors and the IL-8 promoter region [120, 121], and aid in the transcription of CREB targeted genes, many of which regulate metabolic functions such as gluconeogenesis and lipid metabolism [122].

CREB stimulates target gene expression at promoters that contain CREB-response elements (CRE), typically palindromic (TGACGTCA) or half-site (TGACG or CGTCA) sequences. In their basal, phosphorylated state, CRTCs are sequestered within the cytoplasm through interactions with 14-3-3 proteins. Dephosphorylation of CRTCs triggers their nuclear translocation, where their binding to CREB results in increased CREB occupancy of CRE sites and target gene activation [122]. Subsequent investigations showed that tumors characterized by LKB1 loss had enhanced CRTC1 activity. In lung tumors with endogenous LKB1, CRTC1 remained phosphorylated and in the cytoplasm. In contrast, LKB1-deficient tumors showed enhanced nuclear localization of CRTC1, elevated CREB activity, and transcription of CREB-dependent targets that facilitated cell growth [115, 123]. Similar LKB1-dependent effects on CRTC1 were seen in esophageal cancer cells, with the upregulation of CREB genes involved in invasion and metastatic behavior [124]. Lastly, a group of LKB1-deficient lung cancer cell lines contained no phosphorylated CRTC1, with resulting enhanced transcription of the inflammatory mediator COX2. Concordantly, LKB1-deficient lung cancer cell lines selectively responded to COX2 inhibitors when compared to LKB1 wild-type cells expressing phosphorylated CRTC1 [116]. Interestingly, several studies have also implicated LKB1 in the regulation of CRTC orthologs by indirect mechanisms, e.g., through AMPK and another AMPK family member, salt-inducible kinase (SIK). For example, CRTC2 is a direct phosphorylation target of AMPK. Under nutrient deprivation, activated AMPK phosphorylates CRTC2, which sequesters the transcriptional coactivator in the cytoplasm and prevents it from entering the nucleus and aiding CREB in transcription of target genes [125]. Phosphorylation of AMPK by LKB1 further regulates this process in mouse hepatocytes [98].

LKB1-deficient HeLa cells (derived from an LKB1-deficient invasive adenocarcinoma of the uterine cervix) [31] were used to explore the control of CREB via SIK and CRTC1. In the absence of LKB1, SIK was unable to phosphorylate CRTC1, leading to constitutive activation of CREB activity. Overexpression of LKB1 in HeLa cells restored SIK activity and minimized CREB transcriptional activation. Furthermore, treatment of LKB1-expressing HEK293 cells with staurosporine, a

CRTC1 inhibitor, elevated CREB activity [126]. CRTC3 has also been implicated as a SIK substrate in macrophages [127].

A role for the LKB1–CRTC–CREB signaling axis has not been formally established in uterine endometrial cancer. However, CREB does regulate endometrial cell proliferation under various conditions. For example, the Ishikawa endometrial cancer cell line utilizes CREB to transcribe cyclin D1 and promote cell cycle progression in the presence of leptin [128], an adipocyte derived hormone, and regulate the synthesis of bile acids, which are elevated systemically in obese states [129]. As Ishikawa cells express LKB1, they may be an ideal cell line for further studies on the effects of LKB1 loss on CRTC–CREB signaling. In our own investigations, knockdown of LKB1 via shRNA lentiviral transduction in immortalized endometrial epithelial cells resulted in the production of CCL2 (a potent monocyte chemoattractant) [130], a phenomenon also observed following conditional ablation of the *LKB1* gene in mouse endometrium in a mouse model described later [131]. CCL2 production from these LKB1-deficient endometrial cancers promoted tumorigenesis through increased infiltration of tumor-promoting macrophages. Interestingly, CCL2 is transcriptionally regulated by CREB [132–134], thus hinting at the possible role of LKB1 in uterine cancer as a mediator of CREB targets through CRTC.

Transcriptional Regulation of LKB1

The fact that LKB1 is frequently downregulated at the protein level in cancers in the absence of mutations suggests that other mechanisms (both epigenetic and post-translational) are likely to be functionally significant. Computational analyses of the *LKB1* promoter region have shown the presence of multiple estrogen responsive elements (EREs) [135], STAT binding/interferon gamma-activated sequence (GAS) motifs [136], p53 binding sites, activator protein-1 (AP-1) binding sites, and CCAAT/enhancer binding protein (C/EBP) sites [137]. Of these, the former three have been tested for their effect on *LKB1* transcription in vitro.

Estrogen receptor-α (ER-α) acts classically through genomic EREs. In MCF-7 breast cancer cells, binding of ER-α to the *LKB1* promoter region downregulates *LKB1* mRNA and protein, and knockdown of ER-α led to increased promoter activity and LKB1 transcription. The treatment of cells with 17β-estradiol induced the same effects as ER-α [135, 138], confirming a repressive role of estrogen signaling on LKB1 status. Lowered LKB1 expression observed in subsets of human endometrial adenocarcinomas [17] may thus in part be attributed to aberrant estrogen signaling [139], though this has not yet been extensively tested.

The *LKB1* promoter also contains a STAT binding/interferon gamma-activated sequence (GAS), which is active in MCF-7 and MDA-MB-231 cells. Pharmacological activation of STAT with prolactin increased LKB1 transcription and led to increased LKB1 protein levels. Mutation in the binding of the GAS motif inhibited these effects concurrently with prolactin treatment, implicating a role for JAK-STAT signaling in *LKB1* transcriptional regulation [136]. A link between menses, JAK-STAT signaling, LKB1 expression, and endometrial cancer has not been clearly defined.

The discovery of p53 binding sites in the *LKB1* promoter may be of clinical significance in endometrial cancer. In one study, laser-capture microdissection (LCMD) of high-grade endometrial cancer cases revealed a significant positive correlation between *LKB1* and *p53* mRNA levels; i.e., low *p53* and low LKB1 levels were strongly correlated in high-grade endometrial adenocarcinomas. When an *LKB1* luciferase reporter was cloned into an endometrial cancer cell line (ECC-1), modulation of *p53* levels with siRNA dramatically reduced *LKB1* transcription, whereas *p53* overexpression had the reverse effect. Binding of p53 to these sites was validated by chromatin immunoprecipitation. High-grade endometrial cancer primary tumors evaluated in this study showed a strong correlation between p53 and LKB1 protein expression levels [137]. Notably, mutations in *TP53* occur frequently (>70%) in subsets of endometrial cancer characterized by chromosomal instability [19, 140]. Given this information, it will be of interest to further investigate functional interactions between p53 and LKB1 in cancer, particularly endometrial cancer.

Other potential mechanisms of LKB1 transcriptional regulation include methylation at the prominent (2.1 kb) CpG island spanning the *LKB1* promoter region and first exon. Primary papillary breast, testicular, and colorectal carcinoma cases showed *LKB1* promoter hypermethylation at CpG islands. In colorectal cell lines featuring promoter hypermethylation, *LKB1* transcripts were undetectable [13, 14]. Pancreatic carcinoma cell lines show similar phenomena; interestingly, *LKB1* expression in these cell lines can be restored by treatment with the demethylating agent 5-aza-2′-deoxycytidine [141]. Although these studies suggest that *LKB1* promoter hypermethylation can account for LKB1 protein loss in various cancers, this seems to be context dependent. Evaluation of low- and high-grade endometrioid endometrial cancer cases, for example, showed reduced *LKB1* transcripts but no evidence of promoter hypermethylation [137]. The lack of LKB1 protein expression in the HeLa cell line was initially (and erroneously) attributed to *LKB1* promoter hypermethylation [13]. However, subsequent studies found no evidence of *LKB1* CpG island hypermethylation by methylation-specific PCR in any cervical cancer cell line including HeLa or primary cervical tumor samples [31]. To the contrary, HeLa and other cervical cancer cell lines that do not express LKB1 harbor intragenic homozygous deletions, and thus, loss of LKB1 protein is clearly due to these intragenic deletions rather than as a result of epigenetic silencing. Furthermore, deep sequencing of uterine cancers collectively showed few DNA methylation changes in the *LKB1* promoter [19]. Therefore, the changes in *LKB1* expression often seen in cancer likely relate to currently unknown epigenetic mechanisms and not promoter hypermethylation.

Mouse Models of LKB1-Driven Cancers

Genetic analyses of LKB1 in mice have provided numerous insights into the biological roles of LKB1 and provided diverse and experimentally tractable platforms to both generate and test hypotheses (Fig. 7.6). In addition, such genetically

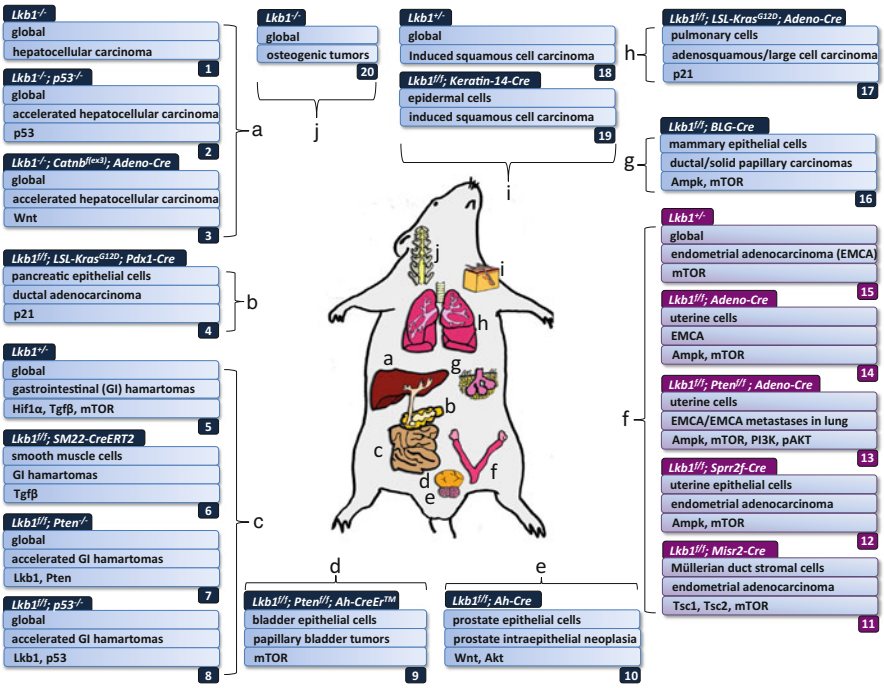

Fig. 7.6 Lkb1 loss induces tumor formation at diverse anatomical sites in murine models. Genotypes of animals and their corresponding tumor sites are grouped as follows: (*a*) liver, (*b*) pancreas, (*c*) GI tract, (*d*) bladder, (*e*) prostate, (*f*) uterus, (*g*) breast, (*h*) lung, (*i*) skin, and (*j*) bone. *Upper dark tabs* denote mouse alleles. *Subsequent tabs* from *top* to *bottom* represent cell type affected, tumor histology, and altered signaling pathways. *Bottom right tabs* indicate publication of the model: (1) Nakau et al. [160]; (2) Takeda et al. [161]; (3) Miyoshi et al. [162]; (4) Morton et al. [163]; (5) Bardeesy et al. [101]; (6) Katajisto et al. [25]; (7) Huang et al. [164]; (8) Wei et al. [165]; (9) Shorning et al. [166]; (10) Pearson et al. [167]; (11) Tanwar et al. [20]; (12) Contreras et al. [18]; (13) Cheng et al. [21]; (14) Contreras et al. [17]; (15) Contreras et al. [17]; (16) McCarthy et al.; (17) Ji et al. [168]; (18) Gurumurthy et al. [169]; (19) Gurumurthy et al. [169]; (20) Robinson et al. [170]. Models pertaining to endometrial carcinoma (discussed more extensively in this review) are shaded *purple*. Figure adapted and updated from a review on mouse models of LKB1-driven cancers by Saara Ollila and Tomi P. Mäkelä, J Mol Cell Biol 2011; 3:330–340) [146]

engineered models (GEMMs) have served as diverse preclinical models to test therapeutic approaches against tumors characterized by LKB1 loss. Nullizygosity for *LKB1* leads to embryonic lethality in mice (e8.5-11, with defects in vasculogenesis and placental development) and hence, biallelic *LKB1* inactivation requires conditional genetic approaches [101, 142]. *LKB1*$^{+/-}$ mice (i.e., genetically similar to individuals with PJS) develop intestinal polyps identical to those seen in PJS. In these mice, the intestinal polyposis is severe, leading to bowel obstruction and early death from the multiple polyps [101]. About half of *LKB1*$^{+/-}$ mice are dead by 40 weeks of age, with 100% mortality by around 55 weeks of age [101]. Efforts to determine if the polyps are due to loss of the second allele have led to different

conclusions and this question remains unresolved, although it appears that at least some individual polyps harbor loss of the second allele [143, 144]. These results are concordant with studies of polyps and gastrointestinal carcinomas in PJS patients, which exhibit LOH in about half of these lesions and occasionally, "second hits" such as mutations in *TP53* [145]. However, it remains possible that the second *LKB1* allele is mutated in some of these lesions by mechanisms other than LOH.

Conditional inactivation of *LKB1* in diverse cell types has yielded a wide range of both tumorigenic and nontumorigenic phenotypes. For the latter, instructive phenotypes have been observed in endothelium, neurons, hematopoietic stem cells, cardiac and skeletal myocytes, hepatocytes, intestinal epithelial cells, and pancreatic β-cells, reflecting the ubiquitous expression of LKB1 and its varied physiologic functions. Tumorigenic phenotypes have also been observed in diverse tissues and cell types, including the liver, mammary gland, pancreas, bladder, prostate, and uterus. The tumorigenic and nontumorigenic phenotypes associated with *LKB1* conditional inactivation are extensively reviewed in [146].

One of the interesting observations from these diverse studies is the striking context dependence of LKB1 as a tumor suppressor. Generally, whereas LKB1 loss is sufficient to drive tumors with high penetrance in some tissues (breast, uterus), LKB1 loss in other tissues (lung, pancreas) results in benign neoplasms (pancreas), preneoplastic phenotypes (prostate), or no tumorigenic or preneoplastic phenotypes at all (lung). Supporting this, biallelic inactivation of *LKB1* in mammary epithelium with a *Cre* transgene under the control of the *β-lactoglobulin* promoter (*BLG-Cre*) led to isolated mammary carcinomas in only 19 % of female mice, strongly suggesting that additional genetic hits were required for tumor formation [147]. In most cell types, simultaneous mutation of a cooperating oncogene or tumor suppressor, such as *KRAS* (lung, pancreas, liver), β-catenin (liver), or *PTEN* (bladder, lung), were required for fully developed malignant phenotypes [146, 148]. In melanocytes, conditional postnatal inactivation of *LKB1* alone did not result in melanocyte hyperproliferation or abnormal pigmentation, whereas simultaneous inactivation of *LKB1* and *KRAS* led to striking melanocytic proliferation, diffuse hyperpigmentation, and biologically aggressive melanomas with high incidence [149]. Not surprisingly, these studies have confirmed that LKB1 is a potent epithelial tumor (carcinoma) suppressor in many tissues, as is evident from the PJS phenotype and its attendant high incidence of carcinomas at multiple sites (Fig. 7.6).

Mouse Models of LKB1-Driven Endometrial Cancers

The most potent tumorigenic phenotypes in LKB1-based mouse models have been observed in the endometrium. Although an initial study of *LKB1*$^{+/-}$ mice reported that occasional female mice harbored benign uterine lesions (i.e., adenomyosis) [101], subsequent investigations revealed that these lesions were in fact extremely well-differentiated endometrioid adenocarcinomas. These highly invasive and lethal cancers were characterized by myometrial infiltration, but their well-differentiated

appearance (recalling the extremely well-differentiated uterine cancers seen in women with PJS) makes them difficult to distinguish from benign lesions such as adenomyosis. In fact, about 50 % of *LKB1*$^{+/-}$ females that did not succumb to gastrointestinal obstruction by 55 weeks of age developed these well-differentiated adenocarcinomas, which were highly stereotypical histologically, and virtually identical histologically across all animals in which tumors arose.

This initial model left unresolved many important questions, such as whether LKB1 functions in a cell-autonomous manner as an endometrial tumor suppressor. To study these and other questions, a second model was generated by direct injection (via the cervical os) of an adenovirus expressing the Cre recombinase (Ad-Cre) into the uterine lumen of female mice homozygous for a floxed *LKB1* allele (*L*). As previously shown, this Ad-Cre approach results in transduction of endometrial epithelium but, because of the presence of tight junctions that prevent tissue penetration of virus, not of endometrial stromal or other cell types within the uterus [150].

Cohorts of 17 *LKB1*$^{L/L}$ homozygous floxed and 30 control wild-type female mice were injected with Ad-Cre at 6 weeks of age and euthanized at 9 months posttreatment. PCR confirmed Cre-mediated recombination and the presence of the *LKB1* null allele in uterine DNA, but not in control tail DNA. Of the 17 *LKB1*$^{L/L}$ mice, 11 (65 %) developed uterine tumors, versus 0/30 of controls mice similarly treated with Ad-Cre ($p = 7.1 \times 10^{-7}$). The majority of these tumors were confined to the uterus, but one was diffusely metastatic within the peritoneum. No extrauterine tumors were observed. Histologically, the tumors in Ad-Cre treated *LKB1*$^{L/L}$ females were identical to those in *LKB1*$^{+/-}$ females, and distant metastatic tumor glands were also essentially indistinguishable from those in primary tumors (i.e., even the metastases were extremely well differentiated). These results confirmed that LKB1 acts as a cell-autonomous tumor suppressor (i.e., inactivation within the epithelium, and *only* within endometrial epithelium, is required for endometrial carcinogenesis) [17].

These LKB1-deficient endometrial cancers were characterized by hypophosphorylation of AMPK and the AMPK target acetylcoenzymeA carboxylase (ACC), demonstrating that AMPK is one important mediator of LKB1 loss in endometrial epithelial cells relevant to tumorigenesis. Given the role of LKB1/AMPK in the establishment of cell polarity in other cell types (discussed earlier) and the fact that epithelial pseudostratification and loss of cell polarity characterizes the majority of even low-grade endometrial cancers, these LKB1-deficient mouse endometrial tumors paradoxically showed no evidence of abnormal polarization either histologically (i.e., nuclei remained basal), ultrastructurally (microvillus morphology and distribution), or by markers of cell polarity (lectin) [17]. Thus, these early models established LKB1 as a *bona fide* tumor suppressor that plays a special role in promoting invasion and also proved useful to explore other questions of biological interest. However, long tumor latency and incomplete penetrance limited their utility. Also, given that Ad-Cre results in recombination with very limited efficiency, it was unclear whether the relatively low observed cancer rate reflected inefficiency of Ad-Cre infection versus the need for additional cooperating oncogenic mutations, as is the case in most human cancers and murine cancer models.

To address these questions and develop an improved model, *LKB1* was conditionally inactivated using *Sprr2f-Cre*, a Cre driver designed to be specifically expressed in endometrial epithelium (i.e., but not in any other uterine compartments, such as endometrial stroma or myometrium) [18]. The *Sprr2f* gene was identified in an expression screen for genes specifically expressed in the endometrium based on a method originally developed for the identification of ovarian-specific genes [151]. *Sprr2f-Cre; LKB1$^{L/L}$* mice were born at expected Mendelian ratios and were externally normal and initially in good health. However, *Sprr2f-Cre; LKB1$^{L/L}$* females exhibited a striking increase in mortality. They began to die as early as 120 days (17 weeks) of age and all were dead in a remarkably short window of time, by 212 days (30 weeks). This mortality was due to invasive endometrial cancers that arose and progressed in a highly stereotypical manner. *Sprr2f-Cre; LKB1$^{L/L}$* uteri developed normally and were of normal weight through the onset of sexual maturity. However, by 16 weeks of age there was significant uterine enlargement, and in females that survived to 28 weeks, average uterine weights were increased tenfold relative to controls due to extensive involvement by invasive, well-differentiated endometrial cancer (Fig. 7.7).

Gross and microscopic examinations confirmed that tumor progression occurred in a stereotypical manner. At 6 weeks of age, *Sprr2f-Cre; LKB1$^{L/L}$* uteri were indistinguishable from sibling controls with no weight increase or microscopic evidence

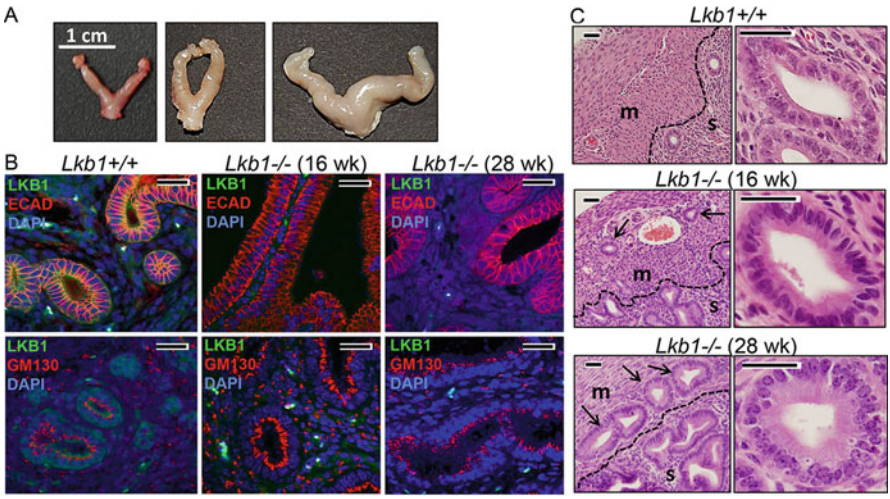

Fig. 7.7 Genetic ablation of *LKB1* induces invasive, well-differentiated endometrial adenocarcinoma. (**a**) Gross *LKB1$^{f/f}$* (control) (*left*), 16 week *Sprr2f-Cre*; *LKB1$^{f/f}$* (*middle*) and 28 week *Sprr2f-Cre*; *LKB1$^{f/f}$* (*right*) uteri. (**b**) Immunofluorescent staining of control, 16 week, and 28 week uteri revealing no changes in lateral (e-cadherin) or apical (GM130) polarity markers in *LKB1$^{-/-}$* epithelium throughout tumor progression. (**c**) Hematoxylin and eosin staining of control, 16 week, and 28 week uteri showing myometrial invasion of *LKB1$^{-/-}$* glandular epithelium (*left, arrows*). High power magnification (*right*) showing invasive *LKB1$^{-/-}$* glands display remarkably well-differentiated histology with no obvious defects in polarity (i.e., nuclei remain basal). E denotes endometrium; m denotes myometrium. Scale bars = 50 μm

of neoplasia or invasion. However, by 12 weeks of age, diffuse infiltration into the myometrium was observed in most animals. This infiltration was progressive throughout the uterus, leading to diffuse uterine enlargement with increasing age due to the growth of tumor and associated stroma (Fig. 7.7). At later time points, invasive endometrial carcinoma spread to adjacent organs, particularly the ovary, cervix, and bladder. The cause of death in most animals was infiltration into the urinary bladder (which lies directly on the anterior aspect of the uterus) with ensuing urinary tract obstruction and hydronephrosis. Invasion through the uterine wall also led to acute peritonitis and sepsis, contributing to morbidity. Distant metastases were observed only occasionally (i.e., one mouse harbored subcutaneous and pulmonary nodules histologically consistent with metastases from the uterine primary). Given these features, along with well-differentiated tumor appearance (Fig. 7.7), this model closely resembles human endometrial adenocarcinoma, which results in morbidity due to local infiltration and spread but rarely metastasizes to distant sites [152].

This refined *Sprr2f-Cre*-based model demonstrated that LKB1 serves unique biological roles and is an extremely significant tumor suppressor gene in the endometrium. The short latency, complete penetrance, diffuse growth pattern, and absence of a definable morphologic precursor are features that together strongly argued that LKB1 inactivation is sufficient for the malignant transformation of endometrial epithelium into invasive adenocarcinoma without the requirement for cooperating oncogenic mutations. Consistent with this interpretation, the uterine tumors were always extremely well differentiated with minimal (if any) nuclear atypia or abnormal mitotic figures (Fig. 7.7), suggesting that widespread genomic instability was not a feature of these tumors [153]. This was confirmed in subsequent investigations, which showed that these LKB1-deficient tumors are diploid or near-diploid unlike other mouse models of endometrial cancer [154]. The early, rapid, stereotypical, and diffuse growth of the tumors led to the conclusion that LKB1 inactivation in endometrial epithelium is sufficient to drive invasive growth. In these respects, this model—in which only one tumor suppressor was inactivated—appears is a rarity in GEMMs of carcinoma, where tumor kinetics and growth patterns have been typically consistent with the requirement for cooperating genetic mutations [155].

This model served as a useful preclinical platform to test the efficacy of rapalogs against LKB1-deficient cancers. Several observations suggested that LKB1-deficient tumors might prove hypersensitive to rapalogs. First, among 690 cancer cell lines of diverse anatomic origin, endometrial cancer cell lines as a group showed the greatest growth inhibition to rapamycin, more so than cell lines of any other cancer type. Second, LKB1 deficiency leads to mTOR hyperactivity, making it likely that LKB1-deficient tumors would be unusually sensitive to mTOR inhibition. For example, rapalog therapy inhibited growth of polyps in $LKB1^{+/-}$ mice [156]. Lastly, mTOR inhibitors such as Temsirolimus led to remissions in a subset of women with advanced endometrial cancer, as previously mentioned.

Notably, in a prophylaxis study conducted with young (12-week old) *Sprr2f-Cre; LKB1*$^{L/L}$ females, 4 weeks of rapamycin therapy led to a significant reduction in tumor burden due to a combination of cytostatic and cytocidal effects.

When rapamycin was administered to mice with large tumors and very advanced disease (imminently requiring euthanasia per compassionate animal use guidelines), tumors rapidly regressed with dramatic responses in overall health. Upon therapy cessation after 6 weeks of treatment, all tumors grew back rapidly. Thus, rapamycin monotherapy not only halted progression of LKB1-deficient tumors but also led to significant and sustained reductions in tumor burden even in animals with very advanced disease, leading to significant lifespan extension and an improved quality of life. These findings suggest that LKB1 status (expression level), or perhaps mTOR pathway status (mutations in *MTOR* or other pathway components) might be predictive of responses to rapalogs in endometrial and other cancers, a question that clearly merits further investigation.

Another study employed the conditional Ad-Cre approach to study genetic interactions between *LKB1* and *PTEN* [21]. Aberrant PI3K signaling is a hallmark of endometrial cancer, and mutations in loci encoding PI3K pathway components (e.g., *PIK3CA* encoding the p110α catalytic subunit, *PIK3R* encoding the p85α regulatory subunit, and *PTEN*) are more common in endometrial cancer than in any other cancer type [19] (see Chaps. 6 and 9). The high frequency of PI3K pathway alterations makes the question of genetic interactions and cooperation between *LKB1* and *PTEN* a subject of interest, especially since mutations in *PTEN* are the most common genetic aberration in endometrial cancer. Consistent with prior studies, endometrial hyperplasias and well-differentiated endometrioid adenocarcinomas were observed in the single-knockout $PTEN^{L/L}$ and $LKB1^{L/L}$ females. However, potent synergism was observed in the double-knockout $PTEN^{L/L}$; $LKB1^{L/L}$ females, as evidenced by accelerated tumor progression and early mortality from the well-differentiated endometrioid adenocarcinomas that arose. Furthermore, macroscopic metastases were identified in 65 % of animals. Phosphorylation of AMPK and ACC was abolished in these tumors, demonstrating misregulation of the LKB1/AMPK axis, while phosphorylation of AKT was increased, as expected from the loss of PTEN [21].

This PTEN/LKB1 endometrial cancer model was then exploited as a preclinical platform to explore the utility of targeted therapies. Strikingly, the dual kinase inhibitor BEZ235, which inhibits both PI3K and mTOR, had a potent antitumor effect in this model. Six weeks of treatment greatly slowed disease progression, with all animals in the treatment arm surviving in the 3-month treatment group (vs. 100 % deaths in the control arm). There was decreased cell proliferation and increased apoptosis in treated tumor cells, with immunohistochemical decreases in pAKT and p-ribosomal protein S6 in tumor epithelium. Additional drug studies were conducted with subcutaneous transplants of *PTEN*/*LKB1* endometrial tumors into immunocompromised hosts (xenografts). Interestingly, not only BEZ235, but the mTOR inhibitor and rapalog RAD001 (Everolimus) completely inhibited *PTEN*/*LKB1* tumor xenograft growth over the ~21 day treatment window. These results suggest that endometrial tumors driven by PTEN and LKB1 loss are highly dependent on mTOR signaling, and further suggest that mTOR inhibitors could be an effective clinical treatment strategy in endometrial cancers (and perhaps other cancers) characterized by PI3K aberrations and low LKB1 expression [21].

The role of LKB1 in the stromal cells of Müllerian derivatives (oviduct, uterus, cervix, and proximal vagina) was explored via a conditional knockout of *LKB1* with the *MISR2* (a.k.a. *AMHR2*/*Anti-Müllerian Hormone Type 2 Receptor*) based Cre driver [20]. The *MISR2* gene is expressed throughout the mesenchyme-derived cells of the murine female reproductive tract, and examination of tissues from *MISR2-Cre* mice bred to the *R26R* β-galactosidase reporter confirmed Cre-mediated recombination only in the stromal compartment (and not the epithelial compartment). Stromal LKB1 loss led to no observable defects in 5-week-old animals (around the time of sexual maturation in mice). However after 18 weeks, the oviducts were abnormal, with stromal (myofibroblastic) cell hyperplasia and disorganization, and cyst formation. Abnormalities in the extracellular matrix (ECM) were observed, including excess collagen deposition. Interestingly, conditional inactivation of *TSC1* or *TSC2* with *MISR2*-Cre phenocopied the defects observed in the *LKB1* mice, arguing that the mTOR pathway is a major effector of these LKB1-driven stromal phenotypes [20].

In the uterus, the *MISR2*-Cre; $LKB1^{L/L}$ mice harbored expansion/overgrowth of the stromal cell compartment but surprisingly also exhibited endometrial epithelial hyperplasia and adenocarcinoma, a phenotype that became more severe with age. These phenotypes were reversible by administration of rapamycin for 3 weeks, demonstrating that mTOR was critical in the development of these LKB1-driven stromal phenotypes and again showing the general feasibility of reversing the effects of LKB1-driven abnormal growth phenotypes pharmacologically. As in the epithelial-specific knockout described earlier, simultaneous inactivation of LKB1 and PTEN in the stroma revealed potent synergistic effects with pronounced tumor growth in the uteri, cervix, and vagina. These studies showed that LKB1/TSC/mTOR signaling in mesenchymal cells is required for the maintenance of epithelial integrity and suppresses carcinogenesis in the adjacent epithelial cells. These results do not contradict the studies demonstrating that *LKB1* has essential roles as a tumor suppressor in epithelium, but rather reveal additional roles of the LKB1/mTOR signaling in stromal cells. They also suggest that tumor-prone phenotypes in PJS patients, who have monoallelic inactivation of *LKB1* in all cells including stroma, may be due to complex interplays of aberrant stromal and epithelial LKB1/TSC/mTOR signaling [20], as suggested by earlier studies of PJS polyps [25].

Lessons from Lkb1 Mouse Models of Endometrial Cancer

In summary, mouse models of LKB1-driven uterine cancer have accelerated research and led to several novel insights. For example, the striking (and somewhat unexpected) endometrial cancer phenotype in the LKB1 endometrial knockout models prompted a systematic analysis of *LKB1* status by MLPA and resequencing in lower reproductive tract cancers, leading to the identification of *LKB1* mutations in cervical cancer several years before the completion of systematic next-generation sequencing analyses [157]. Mouse models have proven useful in the exploration of

genetic cooperativity (e.g., with *PTEN*) and in studying the effects of stromal versus epithelial *LKB1* loss, among other biological questions. In the future, mouse models of other LKB1-dependent gynecologic malignancies—such as SCTAT/granulosa cell tumors, or cervical cancer (including adenocarcinoma, squamous cell carcinoma, and MDA)—would also likely prove to be interesting and valuable models, particularly as tools for the development of such models are available [158, 159]. These models also have special promise for the general goals of therapy individualization and targeted treatment strategies. A consistent finding in all of the LKB1 models of uterine neoplasia is the potent effect of rapalog monotherapy in not only halting but reversing the growth of LKB1-driven uterine tumors. It will be of special interest to determine if the striking clinical responses to rapalogs in a subset of advanced endometrial cancer patients can be predicted by alterations in LKB1/AMPK/TSC/MTOR pathway or its specific components, especially since alterations in the PI3K branch of this pathway have not proven useful in this regard. Clinical trials of rapalogs may also be warranted in LKB1-deficient cervical cancers, where molecular assays (DNA based, etc.) could be employed to identify those LKB1-deficient tumors likely to respond to such therapies.

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Chapter 8
Mig-6 Mouse Model of Endometrial Cancer

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Abstract Endometrial cancer is a frequently occurring gynecological disorder. Estrogen-dependent endometrioid carcinoma is the most common type of gynecological cancer. One of the major pathologic phenomena of endometrial cancer is the loss of estrogen (E2) and progesterone (P4) control over uterine epithelial cell proliferation. P4 antagonizes the growth-promoting properties of E2 in the uterus. P4 prevents the development of endometrial cancer associated with unopposed E2 by blocking E2 actions. Mitogen inducible gene 6 (*Mig-6*, *Errfi1, RALT*, or *gene 33*) is an immediate early response gene that can be induced by various mitogens and common chronic stress stimuli. *Mig-6* has been identified as an important component of P4-mediated inhibition of E2 signaling in the uterus. Decreased expression of MIG-6 is observed in human endometrial carcinomas. Transgenic mice with *Mig-6* ablation in the uterus develop endometrial hyperplasia and E2-dependent endometrial cancer. Thus, MIG-6 has a tumor suppressor function in endometrial tumorigenesis. The following discussion summarizes our current knowledge of *Mig-6* mouse models and their role in understanding the molecular mechanisms of endometrial tumorigenesis and in the development of therapeutic approaches for endometrial cancer.

Keywords *Mig-6* • *Errfi1* • Endometrial cancer • Mouse model • Progesterone • Estrogen

Role of Progesterone in Endometrial Cancer

Progesterone (P4) is one of the steroid hormones produced by the ovaries, and its synthesis and secretion are regulated by luteinizing hormone and chorionic gonadotropin during the menstrual cycle and pregnancy [1]. The P4 responsiveness in the

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endometrium is mediated by the coordinated actions of progesterone receptor (PGR) isoforms A and B [2]. PGR is a major mediator of epithelial-stromal cross-talk through inhibition of 17β-estradiol (E2)-mediated epithelial cell proliferation [1, 3, 4]. Steroid hormonal imbalances can result in abnormal endometrial proliferation which may lead to endometrial adenocarcinoma.

P4 therapy has been used in the conservative endocrine treatment of endometrial complex atypical hyperplasia and early endometrial cancer in young women with a desire to maintain their fertility [5–8]. The survival and proliferation of endometrial cancer can be suppressed by the actions of P4 and its analogs, such as megestrol acetate and medroxyprogesterone acetate, under pathological conditions [9, 10]. However, more than 30 % of patients fail to respond to progestin due to de novo or acquired P4 resistance [7, 11–14]. Further, P4 resistance is seen in a wide variety of endometrial diseases such as infertility, endometriosis, as well as endometrial cancer [15–17]. Therefore, the identification of P4-regulated signaling pathways in the uterus is crucial for understanding the impairments that underlie disruption of steroid hormone control of uterine cell proliferation and differentiation.

MIG-6

Mitogen-inducible gene 6 (*MIG-6*; also known as ERBB receptor feedback inhibitor 1 (*ERRFI1*), receptor-associated Late Transducer (*RALT*), or gene 33 is a 50 kDa cytoplasmic protein whose expression is regulated through mitogenic stimuli in a cell cycle-dependent fashion [18]. It contains 462 amino acids and the gene is located on chromosome 1p36.23. It is widely expressed in the liver, uterus, lung, kidney, heart, and other various tissues [19, 20]. *MIG-6* is an immediate early response gene that can be transcriptionally induced by epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α), as well as stress factors, such as mechanical force [21–26].

MIG-6 is an adaptor molecule containing several important protein–protein interaction domains, a Cdc42- and Rac-interactive binding (CRIB) domain, a src homology 3 (SH3)-binding motif, a 14-3-3-binding domain, and an EGFR-binding domain [21, 27, 28]. However, it does not have any domains with enzymatic activity [26]. MIG-6 acts as a negative feedback regulator of the epidermal growth factor receptor (EGFR) mitogenic function and can suppress ErbB2 oncogenic activity through direct interaction with the EGFR family [24, 29–32].

PGR is critical in the maintenance of pregnancy as well as in the pathogenesis of endometrial diseases such as endometrial cancer and endometriosis. *Mig-6* has been identified as a P4-PGR regulated gene in the mouse uterus using high density DNA microarray analysis and progesterone receptor knock-out mice (PRKO) [33]. Expression of the *Mig-6* gene was significantly increased in the uteri of ovariectomized wild-type mice treated with P4 compared to those exposed only to vehicle. However, its expression was not induced in the PRKO mice treated with P4. *Mig-6* mRNAs and proteins were strongly expressed in the stroma, luminal epithelium,

and glandular epithelium of wild-type mice by P4 treatment. These results suggest that the expression of *Mig-6* in all compartments of the endometrium is regulated by P4 and is dependent upon PGR.

Tumor Suppressor Function of MIG-6 in Other Cancer

Several studies provide evidence for the antiproliferative activity of MIG-6. Downregulated expression of *MIG-6* promotes cell proliferation, migration, and invasion as well as increases the rate of G1-S phase progression [30, 34–38]. MIG-6 is a tumor suppressor in both humans and mice. MIG-6 directly interacts with all members of the EGFR family, including EGFR, ErbB2, 3, and 4, and it acts as a negative feedback regulator of EGFR signaling [24]. Additionally, overexpression or small interfering RNA (siRNA)-mediated knockdown studies have shown the role of MIG-6 as a negative regulator of EGFR signaling [30–32, 36]. Overexpression of *Mig-6* in mouse fibroblasts inhibits several Erbb2-dependent processes, including cell proliferation, transformation, and the durational activation of ERK1/2 [24]. The expression of MIG-6 is decreased in 6 of 9 human breast cancer cell lines and 3 cell lines expressing low levels of MIG-6 exhibited high levels of phosphorylated EGFR [39]. Furthermore, decreased expression of MIG-6 is observed in human breast carcinomas and correlates with reduced overall survival of breast cancer patients. However, mutations in *MIG-6* are not detected in human breast carcinoma [39–41].

The primary hepatocytes isolated from *Mig-6* knockout mice show up-regulation of the EGFR/phosphoinositol 3-kinase/AKT pathway compared with those isolated from wild-type mice. Additionally, MIG-6 is downregulated in human hepatocellular carcinoma and this correlates with increased EGFR expression [37]. The expression of MIG-6 is abundant in all normal thyroid specimens, whereas 77 % of papillary thyroid cancers show low MIG-6 expression due to *MIG-6* promoter hypermethylation [35]. Down-regulation of MIG-6 is associated with low nuclear factor k-light-chain enhancer of activated B cells (NF-kB) activity but high levels of EGFR, Met, and Src phosphorylation in papillary thyroid cancer [35, 42]. MIG-6 is down-regulated in glioblastomas and it leads to increased tumor invasion, whereas the overexpression of MIG-6 decreases proliferation of glioblastoma cells through suppression of EGFR signaling and promotion of ligand-induced receptor degradation [38, 43]. The expression of MIG-6 is decreased in 52 % of human nonsmall cell lung cancer (NSCLC) [44]. Low expression of MIG-6 is correlated with a poor prognosis in patients with lung cancer. Patients with high expression of MIG-6 had a statistically significantly longer survival than those with low expression of MIG-6 [34, 44]. The small interfering RNA (siRNA)-mediated knockdown of *MIG-6* in NSCLC cell lines lead to a significant increase of phosphorylation of AKT, ERK, and EGFR, as well as MMP-2 and MMP-9 [34]. In contrast, MIG-6 overexpression promotes apoptosis and decreases the proliferation and invasive potential of NSCLC cell lines [44]. Additionally, MIG-6 transcriptional silencing due to missense and nonsense mutations in the MIG-6 coding region is found in NSCLC cell lines, as well as in primary human lung cancer [45].

The Role of MIG-6 in Steroid Hormone Regulation

The ovarian steroid hormones P4 and E2 are essential regulators of reproductive events and are associated with all aspects in the establishment and maintenance of pregnancy [3, 46]. In addition, they regulate growth factor communication networks between the uterine stroma and epithelium through their cognate nuclear receptors [47]. E2 stimulates proliferation of both the uterine epithelial and stromal cells in neonatal mice. However, this proliferative action of E2 is restricted to epithelial cells in the adult mouse uterus [48, 49]. P4 is inhibitory to E2-mediated proliferation of the luminal and glandular epithelial cells. P4 achieves this inhibition of proliferation by coordinating stromal–epithelial crosstalk [3, 49–51]. An imbalance caused by increased E2 action and/or decreased P4 action can result in abnormal endometrial proliferation and endometrial adenocarcinoma [52].

Mig-6 is an important mediator of P4 inhibition of E2 signaling in the uterus [33, 53]. Ablation of *Mig-6* in the mouse uterus ($Pgr^{cre/+}Mig\text{-}6^{f/f}$; $Mig\text{-}6^{d/d}$) results in the inability of P4 to inhibit E2-dependent uterine weight gain in mice [33]. $Mig\text{-}6^{d/d}$ and $Mig\text{-}6^{f/f}$ mice responded to E2 treatment with an increase in uterine wet weight. The E2 responsive genes, lactotransferrin (*Ltf*), chloride channel calcium activated 3 (*Clca3*), and complement component 3(*C3*), were significantly increased in the $Mig\text{-}6^{d/d}$ mice as compared to the $Mig\text{-}6^{f/f}$ mice. This indicates that ablation of *Mig-6* did not enhance the effect of E2 treatment alone. However, P4 did not inhibit the E2-induced hypertrophy in $Mig\text{-}6^{d/d}$ mice. Examination of P4 target gene expression showed no change in the ability of PGR to regulate the expression of follistatin (*Fst*) and amphiregulin (*Areg*) in the $Mig\text{-}6^{d/d}$ mouse. This result demonstrates that ablation of *Mig-6* in the uterus results in an increase of E2 sensitivity of the uterus in the presence of P4. Furthermore, MIG-6 expression is significantly increased in the endometrial epithelium of early secretory phase in endometrial tissue from healthy women [33]. These observations support an important growth regulatory role for MIG-6 via regulation of steroid hormone signaling in the uterus of both humans and mice.

The Physiological Function of *Mig-6* in the Endometrium

According to the expression profile of *Mig-6* in mouse uteri during early pregnancy, *Mig-6* expression is increased from 0.5 days postcoitus (dpc) to 5.5 dpc, reaching statistical significance after 2.5 dpc, which correlates with both an increase in serum P4 levels and PGR expression [53, 54]. *Mig-6* expression is also induced in the uterus by acute E2 or P4 treatment, and its induction is synergistically induced by E2 and P4 treatment. Female mice with conditional ablation of *Mig-6* in the *Pgr*-positive cells ($Mig\text{-}6^{d/d}$ mice) are infertile due to an implantation defect [33, 54]. Ovarian function and embryonic development are not affected in $Mig\text{-}6^{d/d}$ females, confirming that the fertility defect seen in $Mig\text{-}6^{d/d}$ mice is primarily of uterine origin. $Mig\text{-}6^{d/d}$ mice significantly increased the estrogen receptor alpha (ESR1)

activity and expression level of E2-responsive genes, *Clca3*, *C3*, *Ltf*, and mucin 1 transmembrane (*Muc-1*), compared with control mice during the preimplantation period [54]. The *Muc-1* is an E2 target encoding an epithelial glycoprotein, and its expression during peri-implantation prevents uterine receptivity and embryo attachment [55, 56]. These findings demonstrate that abnormally increased E2 activity through absence of *Mig-6* is the underlying cause of the uterine receptivity defect.

The Expression of MIG-6 in Human Endometrial Cancer

Endometrial cancer is the most frequently diagnosed malignancy of the female genital tract. Endometrial cancer is closely associated with endometrial hyperplasia, unopposed E2 exposure, and genetic alterations [57, 58]. E2-dependent endometrioid carcinoma is the most common type of gynecological cancer [58, 59]. Over 80 % of endometrial cancers are adenocarcinomas, meaning they originate in uterine epithelial cells. The examination of MIG-6 mRNA and protein expression in the human endometrium during the menstrual cycle revealed that MIG-6 expression in the endometrial epithelium is highest in the early secretory phase of the cycle. These results suggest that the expression of MIG-6 correlates with P4 regulation in human endometrium as observed in the mouse [33]. *Mig-6* ablation shows altered uterine function due to the inability of P4 to attenuate E2 action, which is a common characteristic of endometrial cancer in humans [60, 61]. In order to understand the role of MIG-6 in endometrial cancer, the expression of MIG-6 was examined in women with or without endometrial cancer. The level of *MIG-6* mRNA is significantly decreased in patients with endometrioid carcinoma (32.8 %) compared to normal endometrial biopsies taken from women during the secretory phase of the cycle [33]. Immunohistochemical analysis also shows a decrease in the protein level of *MIG-6* in patients with endometrial cancer compared to normal endometrium [33].

The Development of Endometrial Cancer in Conditional Ablation of *Mig-6*

Since *Mig-6* ablation results in numerous pathologies and decreased longevity [26, 39, 45, 62], our ability to investigate the role of *Mig-6* in the mouse uterus is severely limited. In order to effectively investigate the role of *Mig-6* in the regulation of uterine function and the response to hormonal stimulation, we generated a *Mig-6* conditional null allele, the *Mig-6* flox allele (*Mig-6*$^{f/f}$) [20]. *Mig-6*$^{f/f}$ mice were bred to *Pgr*^{Cre} [63] mice to generate conditional *Mig-6* ablation (*Pgr*$^{cre/+}$*Mig-6*$^{f/f}$; *Mig-6*$^{d/d}$) in the uterus [20, 33]. An increase of the number of endometrial glands and the gland/stroma ratio were observed in the uterus of *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice by histological analysis at 5 months of age similar to the 9-month-old *Mig-6*$^{-/-}$ mouse [20, 33]. These histological changes found in the *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice are consistent with

endometrial hyperplasia seen in human endometrium. Endometrial hyperplasia often precedes the development of endometrioid endometrial carcinoma [64, 65]. It is defined as an increase in the gland-to-stroma ratio when compared with normal proliferative endometrium [66]. Clinicopathologic and epidemiologic studies have supported the malignant potential of endometrial hyperplasia and the concept of a continuum of proliferative glandular lesions culminating, in some cases, in carcinoma [64, 65]. The proliferation of the endometrial epithelium in *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice was significantly increased. The expression of ESR1 and phosphorylation of ESR1 at Ser 118 were also significantly increased in the endometrial glands.

The endometrial hyperplasia phenotype in the *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice support a tumor suppressor role for *Mig-6* in endometrial tumorigenesis. Risk factors for endometrial cancer include obesity, diabetes mellitus, unopposed E2 replacement therapy, use of tamoxifen, and hypertension [67, 68]. The major pathologic phenomenon of endometrial cancer is the loss of ovarian steroid hormone control over uterine epithelial cell proliferation and apoptosis [58, 59]. One of the endocrine risk factors for developing endometrial cancer is unopposed E2, conversely a lower incidence of these diseases in women is associated with decreased endogenous E2 production [60]. E2-dependent endometrial cancer is the most common type of gynecological cancer [48, 69]. The antagonistic effect of P4 on E2 forms the rationale for P4-based therapeutics for endometrial cancers [70]. For this reason, the effect of ovarian steroid hormones on the development of the hyperplastic phenotype observed in *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice was investigated. Ovariectomized *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice did not develop endometrial hyperplasia as observed in intact *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice. All of the *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice treated with E2 for 3 months showed a significant increase in uterine weight and developed invasive endometrioid-type endometrial adenocarcinoma. The neoplastic endometrial glands in the *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice invaded through the uterine muscle wall and invaded adjacent structures such as the colon, pancreas, and skeletal muscle. This demonstrates that the endometrial hyperplasia phenotype of *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice is dependent on ovarian hormone stimulation. Therefore, the pathophysiology of endometrial hyperplasia and endometrial cancer in *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice is similar to humans (Fig. 8.1). These data suggest that *Mig-6* has an E2-dependent tumor suppressor function in endometrial cancer [33].

Tumor Suppressor Function of *Mig-6* Coordinates Endometrial Stromal–Epithelial Communication

Mig-6 is an important mediator of P4 signaling in the uterus. Conservative treatment with high-dose P4 has been attempted in premenopausal women with endometrial cancer who have a strong desire to preserve fertility [14, 71–77]. P4 therapy prevents the development of endometrial cancer associated with unopposed E2 by blocking E2 actions [78]. However, more than 30 % of patients with progestin treatment did not respond to progestin due to de novo or acquired progestin resistance [7, 11–13]. Therefore, determining the tumor suppressor function of P4, acting

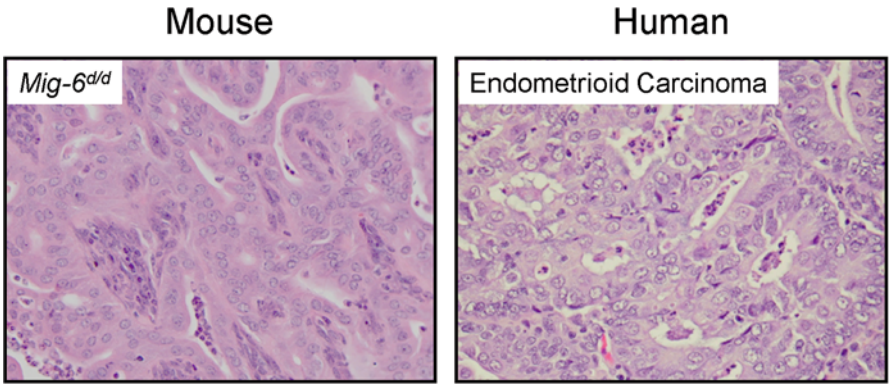

Fig. 8.1 Endometrial cancer in the mouse model and humans

through *Mig-6,* is critical in understanding the role of steroid hormone signaling in endometrial cancer. Epithelial cell-specific *Mig-6* knockout (*Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$) mice were generated to assess the role of epithelial *Mig-6* in tumorigenesis [20, 79]. *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice developed endometrial hyperplasia. In addition, *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice developed E2-dependent endometrial cancer. Interestingly, epithelial proliferation was significantly increased and apoptosis of the subepithelial stroma cells was significantly increased in *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice compared to control mice. NOTCH1 expression was increased in the luminal and glandular epithelium of *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice, whereas it was only expressed in the stromal cells of control mice. In addition, the expression of BIRC3 was increased in the luminal and glandular epithelium of *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice compared to control mice, whereas the expression of BIRC3 was not observed in subepithelial stroma cells of *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice. It is reported that Notch pathway plays an important role in endometrial cancer progression by regulating proliferation [80] and BIRC3 contributes to the survival of endometrial cancer cells against apoptosis mediated by inhibition of AKT [81]. Therefore, these results suggest that the development of endometrial hyperplasia in *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice is due to an increase of epithelial proliferation through BIRC3 and NOTCH1.

In addition, expression of PGR has been studied as prognostic factors for endometrial carcinoma [82–84]. PGR directly interacts with STAT3 [85, 86] and is an essential key regulator of uterine epithelial-stromal crosstalk [87, 88]. The expression of PGR and STAT3 was decreased during endometrial hyperplasia development in the stroma of *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice, meaning dysregulation of STAT3 and PGR crosstalk is important for endometrial hyperplasia development.

P4 inhibits and even reverses E2-induced growth, hyperplasia, or adenocarcinoma of endometrium. P4 exposure is a negative risk factor for endometriosis [70], and pregnancy or progestin-based therapies can lead to disease regression in some women [89, 90]. Progestin has been used in the conservative endocrine treatment of patients with early endometrial cancer in order to preserve their fertility. It is also

used as palliative treatment for patients with advanced stages of endometrial carcinoma [6–8, 91]. Expression of PGR is positively correlated with a good prognosis and response to progestin treatment [92]. P4 therapy prevents the development of endometrial cancer associated with unopposed E2 by blocking E2 actions [78]. However, more than 30 % of patients with progestin treatment do not respond to progestin due to de novo or acquired progestin resistance [7, 11–13]. The mechanism of progestin resistance is still unknown. The hyperplasia phenotype seen in *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice was prevented by P4 treatment while *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice were P4 resistant. A significant decrease of proliferation and an increase of apoptosis were observed in *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice compared to *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ after P4 treatment. The baculoviral inhibitors of apoptosis repeat-containing 1 (*Birc1*), are a family of antiapoptotic proteins [93, 94]. Their expression were significantly decreased in *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice compared to *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice after P4 treatment. ESR1 protein level and its target genes (*Muc-1*, *Clca3*, and *Ltf*) levels were decreased whereas PGR target genes, *Fst* and *Il13ra2* expression were highly increased in the uteri of *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice compared to the uteri of *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ mice after P4 treatment. The expression of BIRC3 was decreased in the epithelium of *Wnt7a*$^{cre+}$*Mig-6*$^{f/f}$ mice while the high levels of BIRC3 was not changed in the epithelium of *Pgr*$^{cre+}$*Mig-6*$^{f/f}$ mice after P4 treatment. These data suggest that P4-induced stromal *Mig-6* can contribute to the prevention of endometrial hyperplasia and that epithelial *Mig-6* is a critical tumor suppressor involved in P4-mediated protection against the development of endometrial cancer [95].

The Synergistic Effect of *Mig-6* and *Pten* Ablation on Endometrial Cancer Development and Progression

PTEN (phosphatase and tensin homolog deleted from chromosome 10) is one of the most frequently mutated tumor suppressor genes in human cancers [96]. Endometrial cancer is associated with mutations in the tumor suppressor gene *PTEN* [57]. *PTEN* is lost or mutated in >50 % of primary endometrioid endometrial cancers [64] and in at least 20 % of endometrial hyperplasia, the precancerous lesions of the endometrium [64, 65]. Loss of *PTEN* is an early event in the multistep process leading to endometrioid endometrial cancer. Previously, loss of *Pten* (either as a heterozygote or by uterine specific ablation) mice develop endometrioid endometrial adenocarcinoma [97, 98]. Since *Mig-6* has an important role as a negative regulator of E2-induced tumorigenesis, the synergistic effect of dysregulation of the *Pten* and *Mig-6* signaling was examined using *Pten* and *Mig-6* ablation in PR-expressing cells (*Pgr*$^{cre/+}$*Mig-6*$^{f/f}$ *Pten* $^{f/f}$ mice). The survival time of *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$*Pten* $^{f/f}$ mice was significantly shorter compared to ablation of either gene alone. *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$*Pten* $^{f/f}$ mice exhibited dramatically accelerated development of endometrial cancer. The *Pgr*$^{cre/+}$*Mig-6*$^{f/f}$*Pten* $^{f/f}$ mice developed endometrial cancer at 4 weeks of age with neoplastic endometrial glands invading through the myometrium. At the same age, the *Pgr*$^{cre/+}$*Pten*$^{f/f}$ mice exhibited only endometrial hyperplasia. A significant

decrease of apoptosis was observed in the epithelium of $Pgr^{cre/+}Mig\text{-}6^{f/f}Pten^{f/f}$ mice at 2 weeks of age whereas the proliferation was not different between $Pgr^{cre/+}Pten^{f/f}$ and $Pgr^{cre/+}Mig\text{-}6^{f/f}Pten^{f/f}$ mice. The decreased epithelial apoptosis may lead to the accelerated tumorigenesis. In addition, the expression of E2-induced apoptotic inhibitors *Birc1* was significantly increased in $Pgr^{cre/+}Mig\text{-}6^{f/f}Pten^{f/f}$ mice compared to control groups. Taken together these data suggest that decreased epithelial apoptosis lead to the accelerated tumorigenesis and *Mig-6* acts as a tumor suppressor in the context of *Pten* ablation by promoting apoptosis through the expression of the *Birc1* family of proteins [99].

Mig-6 Suppresses Endometrial Cancer Associated with *Pten* Deficiency

In order to determine the tumor suppressor function of *Mig-6* in the development of endometrial cancer, conditional overexpression of *Mig-6* mice was generated in $Pgr^{cre/+}Pten^{f/f}$ mice ($Pgr^{cre/+}Mig\text{-}6^{over}Pten^{f/f}$ mice). The survival time of $Pgr^{cre/+}Mig\text{-}6^{over}Pten^{f/f}$ mice was significantly longer than $Pgr^{cre/+}Pten^{f/f}$ mice. While $Pgr^{cre/+}Pten^{f/f}$ mice developed endometrial cancer, $Pgr^{cre/+}Mig\text{-}6^{over}Pten^{f/f}$ mice did not develop endometrial cancer. This result indicates that overexpression of *Mig-6* suppresses endometrial cancer development in the setting of a *Pten* mutation. The proliferation in epithelial cells of $Pgr^{cre/+}Mig\text{-}6^{over}Pten^{f/f}$ mice was significantly lower than in $Pgr^{cre/+}Pten^{f/f}$ mice. The expression of *Hif1α* and its target genes which are rapidly activated by E2 was significantly decreased in $Pgr^{cre/+}Mig\text{-}6^{over}\ Pten^{f/f}$ mice compared to $Pgr^{cre/+}Pten^{f/f}$ mice. These data support that a decrease of proliferation retarded endometrial cancer development and progression in $Pgr^{cre/+}Mig\text{-}6^{over}Pten^{f/f}$ mice via regulating HIF1α signaling. $Pgr^{cre/+}Mig\text{-}6^{over}Pten^{f/f}$ mice showed an increase of PGR protein level in stromal cells and its targets (*Il13ra2* and *Fst*) compared to $Pgr^{cre/+}Pten^{f/f}$ mice at 3 months of age. ESR1 target genes, *Muc-1* and *Ltf* expression were highly decreased in the epithelial cells of $Pgr^{cre/+}Mig\text{-}6^{over}\ Pten^{f/f}$ mice compared to to $Pgr^{cre/+}Pten^{f/f}$ mice. However, ERα protein level was not changed between $Pgr^{cre/+}Pten^{f/f}$ and $Pgr^{cre/+}Mig\text{-}6^{over}\ Pten^{f/f}$ mice. These data support that overexpression of *Mig-6* suppresses endometrial cancer progression by activating P4 signaling and suppressing E2 signaling.

MIG-6 Directly Inhibits Phosphorylation of ERK1/2 Activity

MIG-6 associated proteins were identified to gain insight into its mechanism of action using mass spectrometry of $Pgr^{cre/+}Mig\text{-}6^{f/f}$ mice. 14-3-3 proteins are known as MIG-6-associated proteins [26] that regulate the phosphorylation of proteins involved in PTEN/PI3K/AKT signaling [100, 101]. The molecules such as STAT3, extracellular signal-regulated kinase 2 (ERK2), and growth factor receptor bound

protein 2 (GRB2) were found as novel MIG-6 associated molecules [95]. E2-mediated induction leads to the activation of two key signaling cascades, the PTEN/PI3K/AKT and the ERK pathways [102]. The estrogen receptors (ESR1 and ESR2) mediate the effect of E2 to regulate cellular processes, such as proliferation, apoptosis, and differentiation by transcriptional activation of its target genes [103] or via nongenomic mechanisms which results in the rapid activation of several signal transduction pathways. E2 exerts a proliferative effect via nongenomic activation of ERK1/2 and PI3K/AKT [104]. MIG-6 interacts with ERK2 [99] and directly inhibits phosphorylation of ERK1/2 [105]. $Pgr^{cre/+}Mig\text{-}6^{over}Pten^{f/f}$ mice exhibited an increase of phospho-ERK1/2 and its target genes compared to $Pgr^{cre/+}Pten^{f/f}$ mice. However, the PTEN/PI3K/AKT pathways related proteins and ERK downstream genes were not significantly changed between the mice. U0126 is a highly selective inhibitor of ERK signaling [106]. $Pgr^{cre/+}Pten^{f/f}$ mice showed significantly reduced endometrial tumorigenesis and reduced uterine weight after U0126 treatment. Histopathological analysis of the entire animal cohort showed that inhibition of ERK1/2 phosphorylation suppressed endometrial cancer progression from hyperplasia or normal endometrium in $Pgr^{cre/+}Pten^{f/f}$ mice. These findings suggest that regulation of ERK1/2 phosphorylation is important for the progression of endometrial cancer in *Pten* conditional knock-out mice (Fig. 8.2).

To elucidate the functional role of MIG-6 protein in cellular signaling, in vivo immunoprecipitation assays confirmed that MIG-6 physically interacts with ERK2. The structure and function of MIG-6 and ERK2 with respect to their interaction domains were investigated by mapping the interaction domains of MIG-6 and ERK2. The MIG-6 protein was divided into four fragments, CRIB domain, SH3-binding

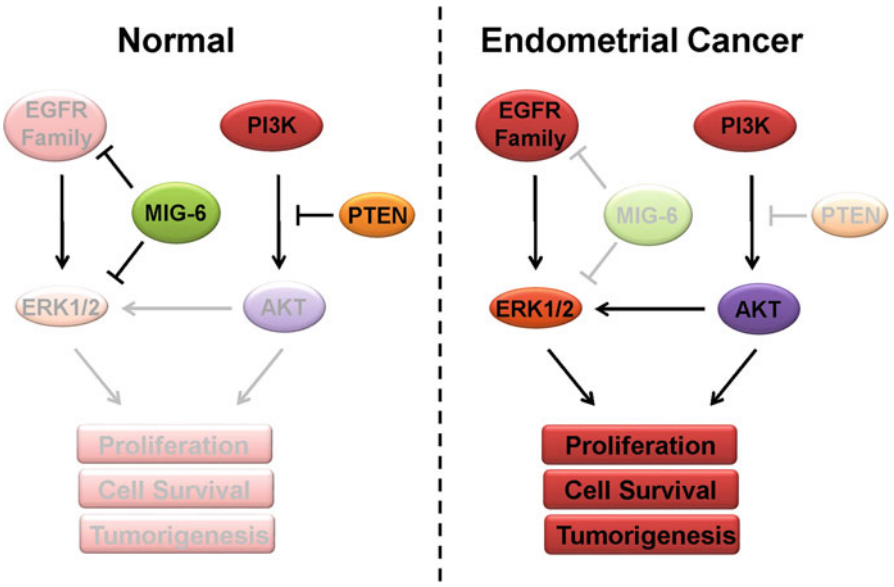

Fig. 8.2 Role of MIG-6 in regulation of ERK1/2 activity in endometrial cancer

domain, a 14-3-3-binding domain, and an EGFR binding domain, and their interaction with ERK2 was confirmed by in vitro pull-down assays. The results showed that the in vitro-translated MIG-6 fragment containing the SH3 binding domain interacted with ERK2. Taken together, these data showed that MIG-6 interacts with ERK2 via its SH3 binding domain. HeLa cells were transfected with Flag-tagged Mig-6, and then in vitro kinase assays were done using GST-ERK2 proteins. Overexpression of MIG-6 decreased the phosphorylation of ERK2. To verify the in vitro kinase assay results, Mig-6-transfected HeLa cell lysates were subjected to western blot analysis using the phosphorylated ERK2 antibody. The phospho-ERK2 antibody was detected in control, but not in the lysates overexpressing-MIG-6. Together, these data establish that MIG-6 inhibits the phosphorylation activity of ERK.

The Clinical Relevance of MIG-6 and ERK1/2 in Human Endometrial Cancer

MIG-6 expression is significantly decreased in grade I, II, and III endometrioid adenocarcinoma compared to normal endometrium. In order to determine the clinical relevance of MIG-6 and ERK1/2 in human, reverse phase protein array (RPPA) was performed in endometrioid endometrial adenocarcinoma. RPPA is a recently developed quantitative assay to analyze nanoliter amounts of sample for hundreds of proteins [107]. MIG-6 expression is inversely associated with ERK1/2 phosphorylation. These results suggest that aberrant overexpression of ERK1/2 phosphorylation is important for tumor development and progression in humans as well as mice.

These studies have established an endometrial cancer mouse model which replicates common characteristics of the human disease providing a model system to further investigate the genetic and molecular events involved in the transition from normal to hyperplastic/neoplastic endometrium. These results will contribute to the understanding of the molecular mechanism of tumorigenesis and to the development of therapeutic approaches for endometrial cancer.

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Chapter 9
PI3K/PTEN/AKT Genetic Mouse Models of Endometrial Carcinoma

Ayesha Joshi and Lora Hedrick Ellenson

Abstract The PI3K/PTEN/AKT pathway is the most frequently mutated pathway in endometrial carcinoma. Mouse models are invaluable tools to understand, at the molecular level, the contributions of components of this pathway towards initiation and progression of endometrial carcinoma. This chapter summarizes results of germline and tissue specific knockout mouse models generated to understand how mutations in components of this pathway lead to development of carcinoma and its interactions with other frequently altered pathways like mismatch repair and estrogen signaling. The mouse models show that loss of both alleles of *Pten* is necessary and sufficient for complex atypical hyperplasia (CAH) to develop but insufficient for progression to carcinoma. Additional events like mutations in *Pik3ca* or mismatch repair deficiency are required for progression to carcinoma. The models show that the interaction between Pten and estrogen signaling is complex. In the absence of estrogen, Pten loss is sufficient for development of CAH. Additionally, lack of ERα on a background of Pten loss leads to the development of carcinoma.

Keywords *Pten* • Mouse models • *Pik3ca* • *Mlh1* • ERalpha

Introduction to Mouse Models

Molecular characterization of uterine endometrial carcinoma (UEC) has revealed that this type of cancer commonly harbors mutations in genes belonging to the PI3K/PTEN/AKT pathway. However, to unravel the mechanistic aspects and cellular functions of these genes in tumor initiation and progression, an in vivo model is crucial. Mice have been the species of choice for modeling many cancers because mouse genomes can be easily manipulated making them amenable to creating complex

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genetic alterations, similar to those found in human tumors. Further, they can also be used to test effectiveness of drugs and targeted therapies in a preclinical setting. It is also possible to generate tissue specific gene alteration using the Cre-Lox system, a methodology useful to understand effects of tumor suppressors and oncogenes, and mutations that would normally cause lethality if deleted or activated in the germline.

Mouse models described below recapitulate human UEC and have provided insight into mechanistic aspects of the most frequently altered pathway in this cancer.

Germline Pten Heterozygous Mouse Model

Pten (Phosphatase and Tensin homolog deleted on chromosome 10) is a key regulatory player in the PI3K/PTEN/AKT pathway. It is a dual phosphatase, and can dephosphorylate both lipids and proteins. Its lipid phosphatase activity plays an important role in the PI3K pathway. Activation of PI3K (phosphatidyl inositol kinase) by growth factor receptors, G-protein coupled receptors and RAS activation leads to generation of PIP3 (phosphatidylinositol 3,4,5 triphosphate) from PIP2 via PDK1 phosphorylation and recruits AKT to the plasma membrane. AKT is a protein kinase that regulates a number of downstream pathways that impinge on cell proliferation, cell growth, and apoptosis. PTEN is a negative regulator of this pathway, acting by converting PIP3 back to PIP2 and inhibiting the activation of AKT and its downstream targets.

UECs harbor the highest frequencies (30–80 %) of intragenic *PTEN* mutations amongst all cancers [1]. Mutations have also been detected in hyperplasia suggesting that in vitro mutations are early events in the pathogenesis. The in vitro gene is encoded by 9 exons. Characterized mutations encompass a wide spectrum including missense, nonsense, and frameshift mutations, which are primarily localized in exons 3, 4, 5, 7, and 8 and target domains involved in protein stability and localization along with the phosphatase domain [2, 3].

The germline *Pten* model was the first genetic mouse model developed to study endometrial carcinoma. The knockout mouse was created by deleting exons 4 and 5 (exons encoding the phosphatase domain) of the *Pten* gene [4, 5]. Due to embryonic expression of *Pten*, the offspring with both copies of *Pten* deleted never survived beyond 6.5 days postcoitum and hence only mice with a single allele of *Pten* deleted ($Pten^{+/-}$) could be analyzed. The $Pten^{+/-}$ genotype displayed neoplasia in multiple organs, including the endometrium.

Analysis of mice uteri starting from 16 weeks up to 40 weeks of age was done to determine the age of onset and progression of the endometrial disease. Light microscopic evaluation of hematoxylin and eosin stained sections displayed endometrial lesions with increasing architectural complexity and cytologic atypia, involving the luminal epithelium and glands (Fig. 9.1b). The lesions were similar to complex atypical hyperplasia (CAH) in humans. The incidence of disease was 100 % by 32 weeks, with multifocal CAH and by 40 weeks of age, 25 % of the mice exhibited carcinoma with stromal invasion. The carcinoma was well differentiated and consisted of cribriform, crowded glands without intervening stroma, recapitulating human UEC. These observations showed that human UEC could be successfully modeled in mice.

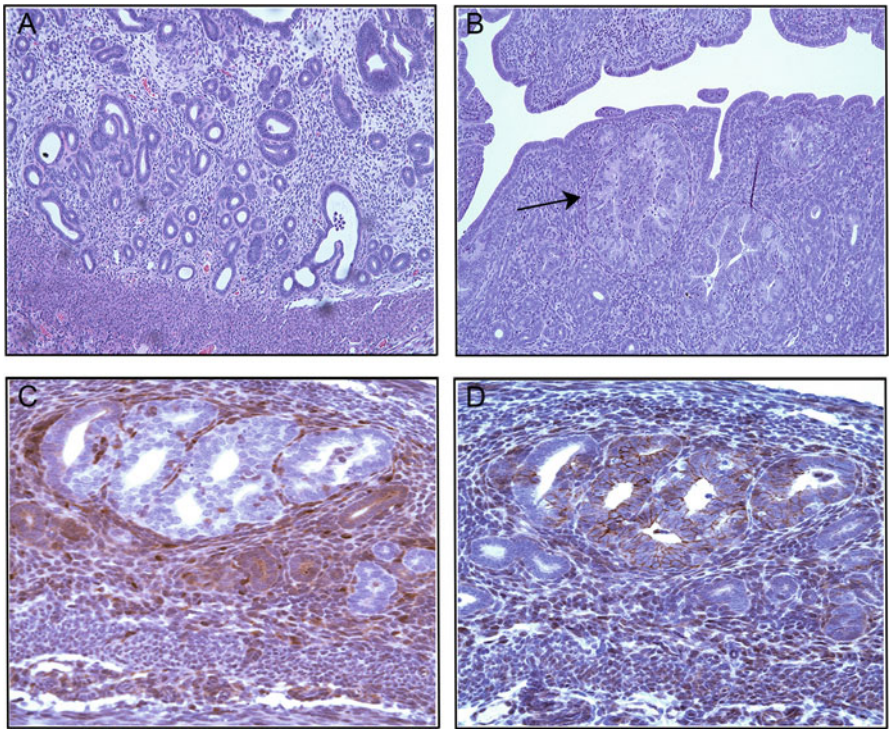

Fig. 9.1 Photomicrographs of hematoxylin-eosin staining of a wild type (**a**) and Pten$^{+/-}$ mouse (**b**) uterus. The wild-type uterus shows presence of normal glandular structures while the Pten$^{+/-}$ uterus shows CAH (*arrow*) with cellular atypia. Magnification 200×. (**c**) Pten immunostaining on a Pten$^{+/-}$ uterus shows an area of CAH with loss of Pten expression while the surrounding stroma and normal glands retain Pten expression, magnification 400×. (**d**) p-Akt immunostaining on the same CAH as in (**c**) showing activation of Akt following Pten loss, magnification 400×. The stroma and normal glands are negative for p-Akt

A striking observation was the complete loss of Pten expression in CAH and UEC when compared to the normal epithelium, as analyzed by immunohistochemistry (Fig. 9.1c). The loss of Pten expression was accompanied by activation of Akt, as evidenced by staining for phosphorylated Akt (p-Akt) in the same lesion (Fig. 9.1d). This suggested that the epithelium lost expression of *Pten* from the normal wild-type allele in all areas with lesions. It was subsequently demonstrated that the loss of expression occurred due to either loss or intragenic mutations of the wild-type allele.

Mlh1 and Pten Mouse Model

Along with PTEN, DNA mismatch repair (MMR) deficiency, as manifested by microsatellite instability (MI), is common in UEC. MI has been detected in approximately 20–45 % of UEC [6, 7] cases and since CAH associated with UEC can also

exhibit MI [8], it is thought to be an early event in the development of UEC. The *MLH1* gene is part of the MMR response in cells. Although no mutations for this gene have been reported in UEC, many sporadic MMR-deficient cases showed reduced mRNA expression in studies including the TCGA study, due to hypermethylation of the *MLH1* promoter [9–11]. Approximately 70–80 % of MI-positive primary tumors also have mutations in *PTEN*, suggesting a link between the MMR and PI3K/PTEN/AKT pathways in the pathogenesis of UEC [12].

Pten$^{+/-}$*;Mlh1*$^{-/-}$ mice were generated to investigate the link between these two pathways in endometrial tumorigenesis [13]. *Pten*$^{+/-}$ mice developed CAH by 16 weeks of age while *Pten*$^{+/-}$*; Mlh1*$^{-/-}$ mice developed polypoid lesions that protruded into the endometrial cavity as early as 6–9 weeks of age. Epithelial cells in these lesions were enlarged and exhibited nuclear atypia, similar to CAH found in *Pten*$^{+/-}$ mice. By 14–18 weeks of age, all *Pten*$^{+/-}$*; Mlh1*$^{-/-}$ mice revealed the presence of lesions histologically identical to CAH and 40 % of mice developed invasive carcinoma (Fig. 9.2a, b). The number and size of lesions was also measured in both *Pten*$^{+/-}$ and *Pten*$^{+/-}$*; Mlh1*$^{-/-}$ mice at the same age (14–18 weeks). *Pten*$^{+/-}$*; Mlh1*$^{-/-}$ mice developed approximately 10 times more lesions and they were also significantly larger than those in *Pten*$^{+/-}$ mice. Of the two animals with invasive disease, one exhibited carcinoma with extensive myometrial invasion with disease extending on to the serosal surface of the uterus. The carcinoma retained glandular differentiation, mimicking well-differentiated invasive tumors in humans. Carcinoma was detected as early as 14–18 weeks, as compared to 40 weeks in *Pten*$^{+/-}$ mice. Thus, although deletion of *Mlh1* alone did not lead to CAH or UEC, when combined with *Pten* loss, it decreased the time of onset and increased the severity of disease. Further, the MI phenotype was detected at U12235 (Fig. 9.2c, d) and MBAT37 mononucleotide repeat tracks in 40 % of microdissected lesions from *Pten*$^{+/-}$*;Mlh1*$^{-/-}$ mice as compared to only 14.3 % in *Pten*$^{+/-}$ mice, confirming MMR deficiency due to *Mlh1* loss. Therefore, MMR deficiency in the setting of *Pten* heterozygosity accelerates endometrial tumorigenesis.

Similar to the *Pten*$^{+/-}$ mouse model, CAH and UEC in the *Pten*$^{+/-}$*; Mlh1*$^{-/-}$ mice exhibited complete absence of Pten expression as determined by IHC analysis. The surrounding stroma and normal epithelium retained expression of Pten. Loss of heterozygosity (LOH) analysis at the *Pten* locus was performed to determine the status of the wild-type allele in Pten-negative lesions. At 14–18 weeks of age, 60 % of lesions in the *Pten*$^{+/-}$*;Mlh1*$^{-/-}$ mice exhibited LOH at the *Pten* locus. This frequency of LOH was observed only in microdissected lesions from 40 week old *Pten*$^{+/-}$ mice. Further, LOH frequency increased from 30 % at 24 weeks to 60 % at 40 weeks in *Pten*$^{+/-}$ mice, suggesting that absence of *Mlh1* accelerated the LOH phenotype on a *Pten*$^{+/-}$ background. Despite lacking Pten expression, 40 % of the lesions did not exhibit LOH, which suggested that the wild-type allele was likely inactivated by other mechanisms. In both the strains however, loss of *Pten* expression from the wild-type allele was an important step in the development of CAH.

To determine the mechanism of *Pten* inactivation in the absence of LOH, all 9 exons of *Pten* were sequenced from the DNA extracted from these lesions. A significant number (37.5 %) of the LOH-negative lesions in the *Pten*$^{+/-}$*;Mlh1*$^{-/-}$ mice showed presence of intragenic mutations in the wild-type allele consisting of deletions of

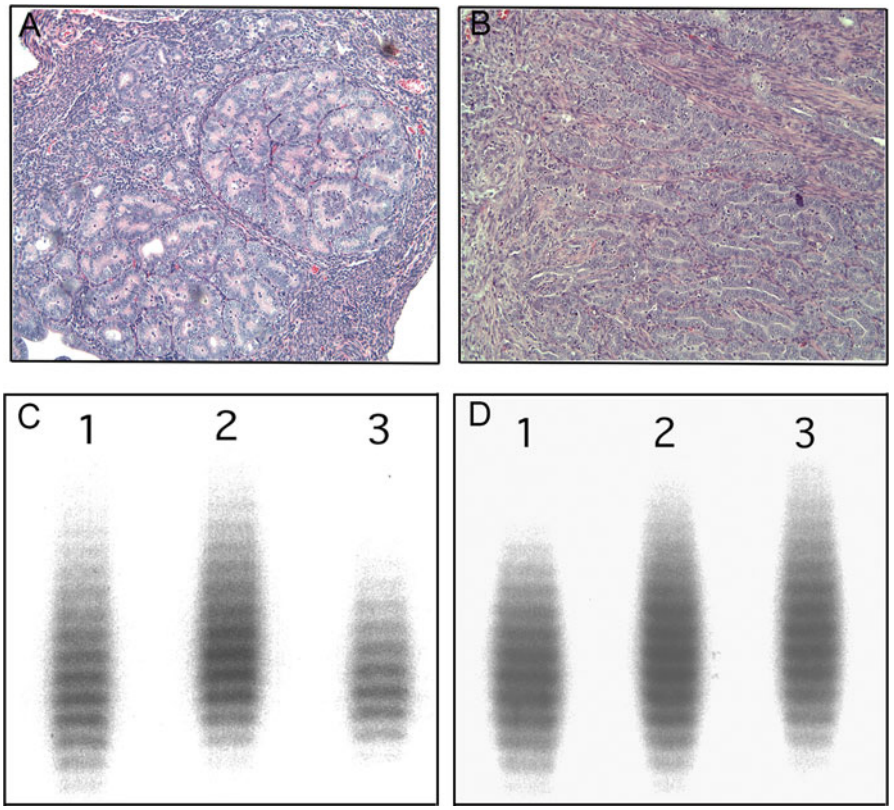

Fig. 9.2 CAH (**a**) and UEC with invasion into the myometrium (**b**) in uteri of $Pten^{+/-}$ $Mlh1^{-/-}$ mouse, magnification 200×. MI analysis on genomic DNA from $Pten^{+/-}$ $Mlh1^{-/-}$ mouse at the U12235 locus (**c**) with *lane 1* showing undiluted tail DNA while *lanes 2* and *3* are the same DNA sample with dilution showing instability. MI analysis of DNA from microdissected lesions from $Pten^{+/-}$ $Mlh1^{-/-}$ mouse at the U12235 locus (**d**) with *lanes 1* showing DNA from normal myometrium while *lanes 2* and *3* showing DNA from CAH lesions with a definitive shift in *lane 3* but not in *lane 2*

poly-A/T tracts at codons 146, 184, and 323. Of note, the deletion at codon 323 has been reported in one primary sporadic human UEC case. LOH-negative lesions from $Pten^{+/-}$ mice however did not harbor intragenic mutations suggesting that the mutations in the *Pten* allele were direct consequences of MMR deficiency. Loss of expression from the wild-type allele in $Pten^{+/-}$ mice might be occurring via yet other mechanisms such as promoter hypermethylation, which has been reported in human tumors. This hypothesis needs further investigation.

The mouse models described above revealed an important relationship between *PTEN* mutation and MMR deficiency in the pathogenesis of endometrial carcinoma and also shed light on the critical role played by *PTEN* in initiation and progression of endometrioid endometrial carcinomas. Although 75–80 % of MI-positive UEC samples also harbor *PTEN* mutations, the spectrum of *PTEN* mutations was similar

in both MI+ and MI- human cases. Further, in the mouse model, *Mlh1*-negative mice lack any endometrial lesions while 100% of *Pten* mutant mice develop CAH. These observations suggest that *PTEN* mutations are not directly attributable to MI in endometrial carcinoma. MMR deficiency in mice accelerated loss of expression from the wild-type allele which in turn accelerated development of CAH in the $Pten^{+/-};Mlh1^{-/-}$ mice as compared to $Pten^{+/-}$ mice. Loss of expression from the wild-type allele may therefore be the rate-limiting step for initiation of the neoplastic process. This observation may explain why women with Cowden's disease are at an increased risk for UEC but show a relatively low disease penetrance.

This mouse model was also used to determine molecular differences between CAH and UEC to identify possible diagnostic markers for use in the clinic [14]. Microarray analysis on DNA from microdissected CAH and UEC lesions identified oviductal glycoprotein gene (Ogp) upregulated eightfold in UEC as compared to CAH. The expression of OGP was tested by immunohistochemical analysis on human CAH and UEC cases. The expression level in all the UEC cases was high. Some weak expression of OGP was also detected in approximately 50% of CAH cases but it was never as intense as that seen in UEC, corroborating the results of microarray analysis. This study indicates that the mouse model can be used to identify diagnostic and potentially prognostic markers in humans (Fig. 9.3).

Although biallelic loss of *Pten* expression was shown to be an important step in the development of CAH, the low frequency of mice developing invasive carcinoma with both *Pten* and MMR deficiency suggested that loss of expression was not sufficient for progression to carcinoma. Epidemiological studies show that loss of *PTEN* expression in CAH on endometrial sampling does not correlate with progression to invasive carcinoma. Hence, although biallelic *PTEN* deletion may be necessary for CAH, progression to UEC requires additional mutational events. It was shown that *PIK3CA*, a gene encoding the catalytic subunit of PI3K, was mutated more frequently in UEC as compared to CAH, while *PTEN* was mutated with equal frequency in both. Thus, acquiring mutations in the *PIK3CA* gene might be one of the mechanisms by which CAH progresses to UEC. PIK3CA and PTEN mutations in uterine endometrioid carcinoma and complex atypical hyperplasia [15].

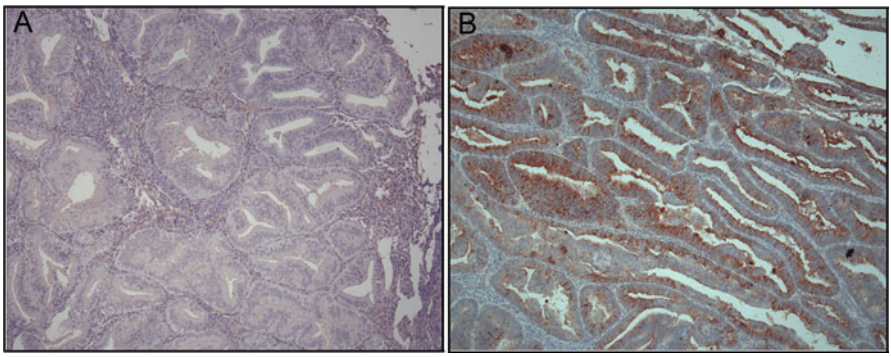

Fig. 9.3 OGP immunostaining on CAH (**a**) and UEC (**b**) from human samples, magnification 200×. Increased OGP staining is seen in UEC as compared to CAH

Tissue Specific Pten Deletion

It became clear with the mouse models described above that biallelic *Pten* deletion was necessary for CAH but was insufficient for progression to carcinoma. However, mice with germline deletion of both *Pten* alleles were embryonically lethal and hence the effect of deleting both alleles in the endometrium could not be analyzed using germline knockout mice. The advent of Cre-lox technology made it possible to restrict deletion of genes in a tissue specific manner [16, 17]. The Cre-lox system was first identified in the P1 bacteriophage viruses. Cre, short for **c**yclization **re**combination, is a DNA recombinase used by the virus to circularize its DNA to facilitate replication after infection of a host cell. The Cre enzyme recombines stretches of DNA flanked by two specific sequences called loxP sequences. Upon encountering loxP sites, the Cre enzyme cleaves the DNA at these sites and re-ligates DNA strands excluding the intervening sequence. This property of the viral enzyme has been adapted for genome manipulation in mammalian cells and is used extensively for creating tissue specific gene deletions [18]. Since mammalian cells and tissues do not express Cre or possess loxP sites, these have to be introduced into the genome. This is achieved by transgenic technology. First, a mouse strain with loxP sequences flanking the region of interest is generated. These strains are called floxed strains. Next, a second strain expressing Cre under a tissue specific promoter is generated. This ensures that the Cre is expressed only in the cells of interest. When the floxed strain is crossed with the Cre strain, recombination and deletion of DNA take place at loxP sites, resulting in gene ablation. Using the technology, the effect of biallelic *Pten* deletion has been studied in many cancer models like breast, colon, brain, prostate etc. This has been possible due to the availability of well-characterized promoters expressed only in these tissues or in a subset of cells within the tissue. A promoter with restricted expression in the endometrium had not been described until recently.

The Cre-lox system can also be used to express mutant alleles of proteins in a tissue specific manner. For instance, mutations in the *PIK3CA* oncogene identified in UEC and other cancers are point mutations that lead to expression of a constitutively active kinase. The endogenous *Pik3ca* allele in the mouse genome is replaced by a mutant allele flanked by loxP sites and is expressed only in the presence of Cre. In this manner, the Cre-lox system can be used to delete as well as activate expression of tumor suppressors and oncogenes in the desired tissue in the mouse.

Mouse Model with Uterine Specific Pten Deletion

The first mouse model with uterine specific *Pten* deletion was generated by crossing *Pten* floxed (designated as $Pten^{f/f}$) mice with mice expressing Cre under the Progesterone receptor (PR) promoter ($PR^{Cre/+}$) [19]. Mice with *Pten* deletion in the uterus exhibited hyperplasia as early as 10 days of age, which progressed to carcinoma by 1 month and developed deep myometrial invasion by 3 months of age and

exhibited 100 % penetrance as compared to the *Pten*$^{+/-}$ germline mouse. The mouse model demonstrated that deleting both copies of *Pten* accelerated the onset and severity of the disease. One caveat of this mouse model is that PR expression is not restricted only to the epithelium in the endometrium. Hence contribution of *Pten* deletion in the stroma, if any, cannot be determined.

Mouse Model with Uterine Epithelium Specific Pten Deletion

To study the effect of deletion of Pten in the epithelium, Cre was expressed under the cadherin 16 promoter. The *Cadherin 16 (Cdh16),* also known as the *Ksp1.3,* gene expression is restricted to the kidney and developing genitourinary (GU) tract. Transgenic *Ksp1.3-Cre* mice express Cre widely in the kidney epithelium [20, 21]. Since the expression of *Ksp1.3* gene was detected in the embryonic GU tract, its expression in the adult uterus was investigated by crossing *Ksp1.3-Cre* transgene to a LacZ reporter strain. In the uterus, the *Ksp1.3-Cre* activity resulted in a mosaic pattern of LacZ expression, present only in luminal and glandular epithelium of the endometrium. The stroma and myometrium did not express *Ksp1.3-Cre* (unpublished results, Joshi et al.). *Pten* floxed (designated as *Pten*$^{f/f}$) mice were therefore crossed to *Ksp1.3-Cre* mice to generate the *Ksp-Cre;Pten*$^{f/f}$ strain [22]. Further, a mutant *Pik3ca* allele with loxP sites (designated as *Pik3ca*^{E545K}) described above was also introduced into the *Ksp-Cre;Pten*$^{f/f}$ strain to create *Ksp-Cre;Pten*$^{f/f}$*;Pik3ca*^{E545K} mice. The E545K mutation is in exon 9 and causes constitutively active Pik3ca. This position has been identified as a hotspot, with high frequency of mutation in UEC as well as other cancers [23]. The uteri of mice from both strains were analyzed at 20 weeks of age. At this age, all the *Ksp-Cre;Pten*$^{f/f}$ mice analyzed exhibited extensive CAH involving the entire luminal epithelium and glands. The surrounding stroma was histologically normal. In some animals, the lesions also exhibited squamous metaplasia. At the same age, 100 % of the uteri from *Ksp-Cre;Pten*$^{f/f}$*;Pik3ca*^{E545K} strain showed carcinoma with invasion into the myometrium. The carcinoma also extended to the ovaries, which were engulfed in cystic structures, lined by malignant cells. Mice with heterozygous *Pten* deletion at the same age showed focal disease and small CAH lesions, similar to the *Pten*$^{+/-}$ germline mouse. Interestingly, heterozygous *Pten* mice with activated mutant *Pik3ca* did not exhibit carcinoma. Of note, mice with activation of *Pik3ca* alone did not develop CAH or carcinoma. Moreover, *Ksp-Cre;Pik3ca*^{E545K} mice had completely normal uterine histology. Primary epithelial cell cultures from these mice showed that mutant *Pik3ca* alone was less effective at activating Akt as compared to *Pten* deletion and might explain the lack of phenotype in these mice. In humans, *PIK3CA* mutations are largely limited to UEC as compared to CAH and the findings from the mouse models may provide some explanation as to why mutant *PIK3CA* may not be sufficient to initiate CAH. The *Ksp-Cre;Pten*$^{f/f}$*;Pik3ca*^{E545K} model has confirmed two important hypotheses. Loss of the second *Pten* allele is the rate-limiting step in the development of CAH and second, biallelic *Pten* deletion is not sufficient for

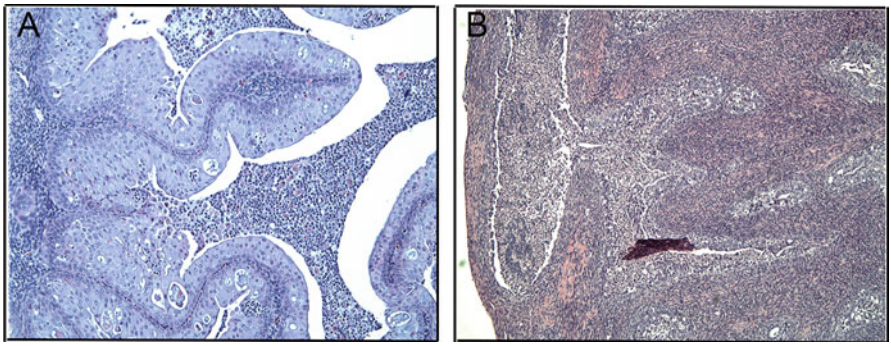

Fig. 9.4 Photomicrographs of hematoxylin-eosin stained sections of *Ksp-Cre;Pten*$^{f/f}$ (**a**) and *Ksp-Cre;Pten*$^{f/f}$*;Pik3ca*^{E545K} (**b**) uteri, magnification 200×. CAH in *Ksp-Cre;Pten*$^{f/f}$ mice show squamous metaplasia while the carcinoma in *Ksp-Cre;Pten*$^{f/f}$*;Pik3ca*^{E545K} shows invasion through the myometrium

progression to carcinoma. Further, it is also clear that *PTEN* is a key tumor suppressor in the endometrium and its loss specifically in the endometrial epithelium is sufficient for the development of CAH (Fig. 9.4).

Interaction Between Estrogen Signaling and PI3K/PTEN/AKT Pathway

UEC is often associated with excess circulating estradiol and low progesterone levels, resulting in unopposed estrogen stimulation [24]. This has also been shown to be true for CAH. Unopposed estrogen, like *PTEN* mutations is considered one of the initiating events, leading to development of CAH and UEC. Several studies have demonstrated an extensive crosstalk between estrogen signaling and the PI3K pathway, particularly in the context of breast cancer [25–27]. AKT and S6 kinase 1 can phosphorylate ERα [27, 28], activating estrogen-independent ER transcription. Estrogen-bound ERα can also bind to the p85 regulatory subunit of PI3K and activate the pathway [26]. Phosphorylation of ERα by Akt was also demonstrated in the *Pten*$^{+/-}$ mouse model [29] and hence, this crosstalk appears to be important in endometrial cancer as well. There is significant evidence for estrogen, acting via ERα to induced growth factor expression in the endometrium as well [24]. The *Pten*$^{+/-}$ mouse model was used to study the effect of high circulating estradiol as well as the role of ERα in the process of tumorigenesis [30].

Pten$^{+/-}$ mice were ovariectomized at 3 weeks of age and sacrificed at 32 weeks. At 32 weeks, the uteri of the ovariectomized mice (*Pten*$^{+/-}$ and wild type) showed ~75 % reduction in weight, as expected due to lack of estrogen. Despite the atrophy, they still developed CAH although the number of CAH foci was reduced as compared to the *Pten*$^{+/-}$ mice with ovaries. Thus, biallelic *Pten* deletion alone can lead to CAH in the absence of estrogen. Additionally, in the setting of a *Pten* mutation,

even physiologic estrogen levels can lead to hyperplasia. Progesterone counteracts proliferative signals from estrogen in the endometrium. Ovariectomized mice also lack progesterone and may explain the hyperplasia although it has been demonstrated that progestin treatment of *Pten*$^{+/-}$ mice does not affect development of endometrial hyperplasia significantly. Hence, reduced progesterone levels may not be a contributing factor to the CAH observed in ovariectomized *Pten*$^{+/-}$ mice.

To mimic the effects of excess estradiol, ovariectomized *Pten*$^{+/-}$ and wild-type mice were implanted with 90-day time-release estradiol pellets, resulting in serum concentrations (200–250 mg/ml) ten times higher than endogenous levels. The pellets were implanted for 12 weeks and a subset of animals were implanted with pellets again for 12 weeks, for a total period of 24 weeks. Animals implanted with placebo pellets served as controls. Three out of the four *Pten*$^{+/-}$ mice treated with estradiol pellets for 24 weeks developed myoinvasive carcinoma. This was in striking contrast to *Pten*$^{+/-}$ mice treated with placebo for 24 weeks or estrogen pellets for 12 weeks, which exhibited only CAH. Interestingly, wild-type mice treated with estrogen pellets for 24 weeks developed dilated complex hyperplasia without atypia. Of note, the number and size of CAH foci in Pten$^{+/-}$ mice treated with placebo or 12 weeks estradiol pellets did not differ significantly. This suggested that estradiol accelerated the onset and increased the incidence of carcinoma but had no impact on the development of CAH. These observations lent support to the hypothesis that biallelic *Pten* inactivation is insufficient for progression of CAH to carcinoma and requires additional events. These events could be either acquiring additional mutations (like *Pik3ca* or *Trp53*) and/or a physiological situation of unopposed estrogen stimulation (Table 9.1).

In the endometrium, ERα is the predominant estrogen receptor and it has been established that estrogen acts on the epithelium directly and indirectly through the stroma. Estrogen signaling in the stroma via ERα leads to secretion of growth factors by the stromal cells, which in turn stimulate epithelial cell proliferation. To dissect out the role of ERα in endometrial tumorigenesis, *Pten*$^{+/-}$ mice were crossed with *ERα*$^{+/-}$ mice. All female mice with *ERα*$^{-/-}$ alleles irrespective of the *Pten* status had hypoplastic uteri as expected due to absence of estrogenic signals. Mice with wild-type *Pten* status did not develop any disease. At 32 weeks of age, CAH was present in all the *Pten*$^{+/-}$*; ERα*$^{+/+}$ and *Pten*$^{+/-}$*; ERα*$^{+/-}$ without any significant difference in the size or number of CAH foci. The *Pten*$^{+/-}$*;ERα*$^{-/-}$ mice exhibited atrophic epithelium but eight

Table 9.1 Incidence, number, and size of neoplastic endometrial lesions in *Pten*$^{+/-}$*;Mlh1*$^{-/-}$ Mice

Pten Genotype	Mlh1 Genotype	Age (weeks)	*n*	No.(%) of mice with lesions	No.(%) of mice with CA	No. of lesions per mouse (mean ± SD)	Size of lesion (mm^2) (mean ± SD)	Range of size (mm^2) (mean ± SD)	LOH (%)
+/–	–/–	6–9	7	6 (85.7)	0	3.43 ± 2.99	NA	NA	NA
+/+	–/–	6–9	4	0	0	0			
+/–	+/+	6–9	5	0	0	0			
+/–	–/–	14–18	5	5 (100)	2(40)	12.20 ± 9.09	0.98 ± 2.39	0.04 → 12	60
+/+	–/–	14–18	5	0	0	0	0		

CA carcinoma, *NA* not analyzed

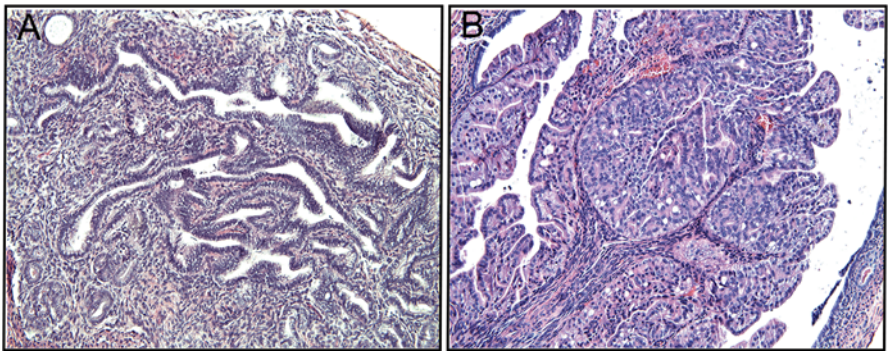

Fig. 9.5 Hematoxylin-eosin stained sections of $Pten^{+/+};ER\alpha^{-/-}$ (**a**) and $Pten^{+/-};ER\alpha^{-/-}$ (**b**) mouse uteri, magnification 200×. $Pten^{+/+};ER\alpha^{-/-}$ uteri exhibit atrophy while the uteri of $Pten^{+/-};ER\alpha^{-/-}$ mice show carcinoma

out of nine mice also developed CAH and/or carcinoma. Notably, four out of the eight $Pten^{+/-};ER\alpha^{-/-}$ mice with endometrial lesions showed in situ carcinoma or carcinoma with invasion into the myometrium. In humans, CAH and grade 1 UEC are generally ERα positive while high-grade tumors are ERα negative. Also, ERα-negative tumors have poor prognosis. The mouse model suggests that reduction in ERα expression may play a role in the progression of the disease and may not be a consequence of decreasing tumor differentiation. However, the majority of estrogen signaling takes place via stromal *ERα*. The $Pten^{+/-};ER\alpha^{-/-}$ mice lack ERα in the stroma as well and carcinoma in these mice may be due to lack of stromal receptor. The contribution of ERα in the stromal cells to the process of tumorigenesis needs to be investigated further. As with *Pten*, floxed *ERα* alleles crossed with *Ksp1.3-Cre* strain will help elucidate the role of this receptor in endometrial carcinogenesis (Fig. 9.5).

These studies highlight the complex interaction between hormones and genetics in the development of UEC. The finding that biallelic *PTEN* inactivation can cause CAH in the absence of estrogen may explain why women without clinical evidence of unopposed estrogen develop CAH. On the other hand, excess estrogen in the setting of *PTEN* mutations may hasten the progression to carcinoma, which may have clinical ramifications for the treatment of *PTEN*-deficient endometrial hyperplasia in patients with Cowden disease.

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