Two-Way Development of the Genetic Model for Endometrial Tumorigenesis in Mice: Current and Future Perspectives

Yoshiaki Maru and Yoshitaka Hippo

Department of Molecular Carcinogenesis, Chiba Cancer Center Research Institute, Chiba, Japan

Endometrial cancer (EC) is the most common malignancy of the female reproductive tract worldwide. Although comprehensive genomic analyses of EC have already uncovered many recurrent genetic alterations and deregulated signaling pathways, its disease model has been limited in quantity and quality. Here, we review the current status of genetic models for EC in mice, which have been developed in two distinct ways at the level of organisms and cells. Accordingly, we first describe the in vivo model using genetic engineering. This approach has been applied to only a subset of genes, with a primary focus on Pten inactivation, given that PTEN is the most frequently altered gene in human EC. In these models, the tissue specificity in genetic engineering determined by the Cre transgenic line has been insufficient. Consequently, the molecular mechanisms underlying EC development remain poorly understood, and preclinical models are still limited in number. Recently, refined Cre transgenic mice have been created to address this issue. With highly specific gene recombination in the endometrial cell lineage, acceptable in vivo modeling of EC development is warranted using these Cre lines. Second, we illustrate an emerging cell-based model. This hybrid approach comprises ex vivo genetic engineering of organoids and in vivo tumor development in immunocompromised mice. Although only a few successful cases have been reported as proof of concept, this approach allows quick and comprehensive analysis, ensuring a high potential for reconstituting carcinogenesis. Hence, ex vivo/in vivo hybrid modeling of EC development and its comparison with corresponding in vivo models may dramatically accelerate EC research. Finally, we provide perspectives on future directions of EC modeling.

Keywords: genetically engineered mouse, organoid, endometrial cancer, carcinogenesis, mouse model, immunodeficient mice

INTRODUCTION

Endometrial cancer (EC) is the most common gynecological malignancy and the fourth most common cancer in developed countries (Yamagami et al., 2017; Siegel et al., 2020). Risk factors for EC include age, obesity, diabetes, and exposure to unopposed estrogen or tamoxifen. The 5-year overall survival rate is between 74 and 90% for patients with stage I or II EC according to the International Federation of Gynecology and Obstetrics (FIGO) staging, whereas that of patients with EC in stage IV EC is 20–26% (Morice et al., 2016). Patients with the following hereditary diseases are...
susceptible to EC development: Cowden’s disease, Peutz–Jeghers syndrome, and Lynch syndrome, which are caused by germline mutations in PTEN (Blumenthal and Dennis, 2008), LKB1 (Hearle et al., 2006), and mismatch repair genes (Meyer et al., 2009), respectively.

According to the traditional classification, EC is roughly divided into two distinct subtypes based on clinical and pathological characteristics. Type I EC, comprising 80% of all ECs, is mainly associated with estrogen dependency, endometrial hyperplasia, and a favorable prognosis. The predominant histological subtype is endometrioid carcinoma, harboring mutations most frequently in PTEN, followed by PIK3CA, KRAS, ARID1A, and CTNNB1. Type II EC, comprising various minor subtypes including serous carcinoma, clear cell carcinoma, and carcinosarcoma (CS), is associated with estrogen independence, endometrial atrophy, and poor prognoses. Mutations in TP53, PIK3CA, PPP2R1A, and ERBB2 amplification are among the most frequent genetic alterations (Kandoth et al., 2013; Murali et al., 2014). Based on comprehensive genomic profiling of EC, four molecular subtypes have been recently proposed: DNA polymerase epsilon (POLE) ultramutated type, microsatellite instability (MSI) hypermutated type, copy number low type, and copy number high type. Patients with the first two subtypes have a significantly better prognosis than those with the latter two subtypes (Kandoth et al., 2013).

As a therapeutic option for EC, surgery is primarily selected if the tumor is locally confined. Surgery is followed by adjuvant radiotherapy or chemotherapy for high-risk patients, such as those suffering from recurrent and metastatic disease, Stage III–IV disease, or type II EC. In these cases, cisplatin, carboplatin, and paclitaxel are the representative agents (Morice et al., 2016). Although molecular targeted therapies, including anti-angiogenesis agents and immune checkpoint inhibitors, are effective in a subset of patients with advanced or recurrent disease (Lee, 2021), treatment options for EC are still limited, underscoring the need for the development of new therapeutic options. Cancer models that accurately mimic the biological properties of human tumors are essential for efficient drug discovery and elucidation of the mechanisms of carcinogenesis. To this end, various murine in vivo models for EC have been established using the genetically engineered mouse (GEM) approach (Van Nyen et al., 2018). Earlier studies have mainly investigated the effect of Pten deletion, while the relevance of other genetic alterations remains to be elucidated.

In this review article, we review the status of genetic models for EC. Specifically, we stratified GEMs by assessing a series of Cre transgenic mice generated to achieve uterine-specific genetic engineering. We also illustrate recent advances in the development of cell-based ex vivo/in vivo hybrid models for EC and provide future perspectives in EC modeling.

**IN VIVO MOUSE MODELS OF EC**

**Background**

The anatomy and physiological function of the female reproductive tract (FRT) differ significantly between humans and mice. In humans, the uterus is a single-lumen organ located in the middle of the pelvis. The two oviducts, also known as fallopian tubes (FTs), protrude from both sides of the uterus and are functionally connected with the ovaries via the infundibulum and fimbrae. In mice, the uterus consists of two uterine horns, and the oviducts are coiled under them inconspicuously. In humans, the reproductive cycle, called the menstrual cycle, lasts for approximately 28 days. The endometrium undergoes cyclical growth, differentiation, and shedding during that period, under the regulation of estrogen and progesterone. In contrast, this cycle in mice, called the estrous cycle, lasts approximately 4–5 days, comprising proestrus, estrus, metestrus, and diestrus periods, without the massive shedding of endometrial tissues into the lumen. Therefore, it is vital to consider these inherent inter-species differences when interpreting the phenotypes of FRT in mouse models.

The generation of GEMs has been the gold standard for modeling tumor development in a physiological setting. Cre-LoxP technology enables the spatiotemporal regulation of gene recombination, further extending its application to the fine-tuning of carcinogenesis models (Deng, 2014; Kim et al., 2018). However, there are some drawbacks to the GEM approach. First, time-consuming and labor-intensive work is often required, especially when conditional alleles are necessary for multiple genes. Second, most current Cre transgenic lines provide limited tissue specificity in generating EC models by intercrossing GEM with conditional floxed alleles. To develop an ideal Cre transgenic line, identifying genes specifically transcribed in endometrial cells is required. Despite intensive efforts, research in the field has not come up with such long-sought mice until recently.

**Mice With Systemic Genetic Engineering**

Pten-null mice die during embryogenesis, clearly indicating their requirement for normal development. Pten+/− mice are born healthy but develop precancerous lesions or malignant neoplasms in multiple organs, including the endometrium, thyroid, and breast (Podsypanina et al., 1999; Stambolic et al., 2000). All Pten+/− mice developed endometrial hyperplasia with various degrees of atypia, 22% of which eventually progressed to EC within a year. Notably, the tumors exhibited loss of heterozygosity (LOH) at the Pten locus, confirming the tumor suppressor role of Pten in EC development. Consistent with the high frequency of MSI in EC (Black et al., 2006; Bonneville et al., 2017), the concurrent Mlh1 loss in Pten+/− mice accelerates endometrial tumorigenesis, highlighting the critical role of DNA mismatch repair deficiency in EC development (Wang et al., 2002). To recapitulate the situation in middle-aged women with unopposed estrogen, 17β-estradiol pellets were implanted in Pten+/− mice that underwent oophorectomy. As predicted, this treatment resulted in increased cancer incidence, underscoring the pro-tumorigenic effects of estrogen. In contrast, Pten+/−: ERα−/− mice paradoxically showed a higher incidence of in situ and invasive carcinoma than Pten+/− mice, suggesting that loss of the ERα could promote EC development. These opposing observations suggest that the effects of Pten inactivation and the estrogen/ERα axis might exert context-dependent effects in the development of EC, pointing toward a narrow window for achieving the right tumorigenic effects (Joshi et al., 2012). For genes other than Pten, there is little information on systemic GEMs with EC development. Therefore, whether a certain combination of systemic genetic alterations could lead to preferential tumor development in the uterus remains largely unknown.
TABLE 1 | Transgenic Cre mice used in generating genetically engineered mice for endometrial cancer models.

| Cre line         | Targeted gene | Penetrance | Latency | EC type | Major histological type | Reference                      |
|------------------|---------------|------------|---------|---------|-------------------------|--------------------------------|
| Pgr-Cre          | Ptenfl/fl     | 100%       | 18-39w  | -       | CAH                     | Poddypypatina et al. (1999)    |
| Pgr-Cre          | Ptenfl/fl     | 21%        | 30-66w  | I       | AC (poorly diff.)       | Stambolic et al. (2000)        |
| Pgr-Cre          | Ptenfl/fl     | 40%        | 14-18w  | I       | AC (well diff.)         | Wang et al. (2002)             |
| Pgr-Cre          | Ptenfl/fl     | 25%        | 32w     | I       | AC                      | Joshi et al. (2012)            |
| Pgr-Cre          | Ptenfl/fl     | 89%        | 12w     | I       | AC                      | Gao et al. (2017)              |
| Pgr-Cre          | Ptenfl/fl, KrasL5-SL-G12D+/− | 100%   | 4w      | I       | AC (EMC)                | Kim et al. (2010)              |
| Pgr-Cre          | Ptenfl/fl, Tgfbr1fl/fl | 100%   | 16w     | I       | AC                      | Gao et al. (2017)              |
| Pgr-Cre          | Ptenfl/fl     | N.A.      | N.A.    | I       | AC                      | Monsivais et al. (2019)        |
| Pgr-Cre          | Ptenfl/fl     | 100%       | 34w     | I       | AC (EMC)                | Kristman et al. (2019)         |
| Pgr-Cre          | Ptenfl/fl     | 47%        | 48w     | II      | AC                      | Stoddard et al. (2013)         |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 12w     | II      | AC (poorly diff.)       | Wang et al. (2002)             |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 30w     | I       | AC                      | Contreas et al. (2010)         |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 43-58w  | I       | AC                      | Peña et al. (2015)             |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 60w     | II      | AC                      | Akbay et al. (2013)            |
| Pgr-Cre          | RhoAfl/fl     | 100%       | 40-67w  | II      | CS                      | Cuevas et al. (2019)           |
| Pgr-Cre          | Pol2fl/fl     | 100%       | 3w      | II      | CS                      | Li et al. (2023)               |
| Pgr-Cre          | R26-PkdcaFlOx | 100%       | 20w     | I       | CAH (moderately to poorly diff.) | Wilson et al. (2019) |
| Pgr-Cre          | R26-PkdcaFlOx | 84%        | 65-79w  | II      | AC (SC, OCC), CS, Undiff. Ca | Wild et al. (2012) |
| Pgr-Cre          | Pax8fl/fl     | 100%       | 6-12w   | I       | EC (EMC)                | Suryo Rahmarto et al. (2020)   |
| Pgr-Cre          | Pax8fl/fl     | 81%        | 57w     | II      | AC (SC)                 | Fu et al. (2020)               |
| Pgr-Cre          | Pax8fl/fl     | 100%       | 6-8w    | -       | AC in situ              | Mirantes et al. (2013)         |
| Pgr-Cre          | Pax8fl/fl     | 90%        | 22-56w  | -       | Stromal tumor           | Kun et al. (2020)              |
| Pgr-Cre          | Pax8fl/fl     | 41%        | 16-32w  | I       | AC                      | Joshi and Elsenor (2011)       |
| Pgr-Cre          | Pax8fl/fl     | 88%        | 2w      | I       | CAH, AC (EMC)           | Saito et al. (2011)            |
| Pgr-Cre          | Pax8fl/fl     | 100%       | 8-28w   | I       | AC (EMC)                | Cheng et al. (2014)            |
| Pgr-Cre          | Pax8fl/fl     | 50%        | 14w     | I       | AC (EMC)                | Tirolkar et al. (2014)         |

FRT, female reproductive tract; N.E., not examined.

TABLE 2 | Past literature on genetically engineered mice for endometrial cancer models.

| Cre line         | Targeted gene | Penetrance | Latency | EC type | Major histological type | Reference                      |
|------------------|---------------|------------|---------|---------|-------------------------|--------------------------------|
| Pgr-Cre          | Ptenfl/fl     | 100%       | 18-39w  | -       | CAH                     | Soyal et al. (2005)            |
| Pgr-Cre          | Ptenfl/fl     | 21%        | 30-66w  | I       | CAH                     | Kontreas et al. (2010)         |
| Pgr-Cre          | Ptenfl/fl     | 40%        | 14-18w  | I       | CAH                     | Cuebas et al. (2019)           |
| Pgr-Cre          | Ptenfl/fl     | 25%        | 32w     | I       | CAH                     | Wang et al. (2002)             |
| Pgr-Cre          | Ptenfl/fl     | 89%        | 12w     | I       | CAH                     | Joshi et al. (2012)            |
| Pgr-Cre          | Ptenfl/fl     | N.A.      | N.A.    | I       | CAH                     | Gao et al. (2017)              |
| Pgr-Cre          | Ptenfl/fl     | 100%       | 34w     | I       | CAH                     | Monsivais et al. (2019)        |
| Pgr-Cre          | Ptenfl/fl     | 47%        | 48w     | II      | CAH                     | Kristman et al. (2019)         |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 12w     | II      | CAH                     | Wang et al. (2002)             |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 30w     | I       | CAH                     | Contreas et al. (2010)         |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 43-58w  | I       | CAH                     | Peña et al. (2015)             |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 60w     | II      | CAH                     | Akbay et al. (2013)            |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 40-67w  | II      | CAH                     | Cuevas et al. (2019)           |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 48w     | II      | CAH                     | Li et al. (2023)               |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 60w     | II      | CAH                     | Li et al. (2023)               |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 43-58w  | I       | CAH                     | Peña et al. (2015)             |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 60w     | II      | CAH                     | Akbay et al. (2013)            |
| Pgr-Cre          | Lkb1fl/fl     | 100%       | 40-67w  | II      | CAH                     | Cuevas et al. (2019)           |

Mice With Uterine-Specific Genetic Engineering

Introduction

Various Cre transgenic mice have been generated to achieve gene recombination in the uterus (Table 1), with varying magnitudes of tissue-specific Cre expression. Nonetheless, certain combinations of genetic aberrations are predisposed to EC development. We illustrate representative conditional GEM models for ECs sorted by Cre transgenic mouse lines (Table 2).

Pgr-Cre Mice

The progesterone receptor (Pgr) is expressed in the uterus and widely across the FRT. Transgenic Pgr-Cre mice expressing Cre under the control of the Pgr promoter (Soyal et al., 2005) have been most commonly used in the generation of GEM for EC models, despite its incomplete tissue specificity (Table 1). As Pgr expression is observed in all major types of cells in the uterus, including epithelium, myometrium, and stroma, floxed genes are deleted in both epithelial and non-epithelial components of the uterus in Pgr-Cre mice. Therefore, genetic aberrations are not
recapitulated exclusively in endometrial cells in mice, unlike in sporadic cases of EC in humans. Pgr-Cre; Pten$^{fl/fl}$ mice frequently develop invasive type I EC by 3 months of age (Daikoku et al., 2008), suggesting that the endometrium is most susceptible to tumorigenesis by Pten inactivation in FRT organs. Kras$^{S12D}$ accelerates Pten inactivation-dependent EC development, underscoring the oncogenic role of mutant Kras in the endometrium (Kim et al., 2010). In the pathogenesis of human cancers, the transforming growth factor β (TGFβ) signaling pathway has multifaceted roles, either pro- or anti-tumorigenic, depending on the organ and genetic context (Gordon and Blobe, 2008). Consistent with this notion, Pgr-Cre; Tgfbr1$^{fl/fl}$; Pten$^{fl/fl}$ mice develop metastatic EC (Gao et al., 2017). It was later demonstrated that even Pgr-Cre; Tgfbr1$^{fl/fl}$ mice develop estrogen-dependent ECs with lung metastasis (Monsivais et al., 2019). Furthermore, Pgr-Cre; Smad2$^{fl/fl}$; Smad3$^{fl/fl}$ mice develop endometrial hyperplasia, which eventually progresses to bulky endometrioid carcinoma with complete mortality by 8 months of age (Kriseman et al., 2019). Based on these results, it is likely that inactivation of the TGFβ signaling pathway plays a pro-tumorigenic role, at least in the pathogenesis of type I ECs. Some GEMs that recapitulate type I EC have also been established. Some GEMs that recapitulate type II EC have also been established.

To recapitulate the highly endometrium-specific transcriptional regulation of Sprz2f in mice, the bacterial artificial chromosome (BAC) clone RP23-3914 was introduced to drive Cre expression instead of the shorter promoter sequence used in Sprz2f-Cre mice. Since the BAC clone harbors a 189 kb genomic fragment spanning the Sprz2 tandem gene cluster region on chromosome 3, it is postulated to be long enough to recapitulate all endogenous transcriptional regulation accurately. Indeed, BAC-Sprz2f-Cre transgenic mice allowed gene recombination in a highly endometrium-specific manner. EC has the highest incidence of FBXW7 mutations among all human cancers, followed by colon cancer (Yeh et al., 2018). BAC-Sprz2f-Cre; Pten$^{fl/fl}$; Fbxw7$^{fl/fl}$ mice developed endometrioid carcinoma, which eventually progressed to CS at 40–67 weeks. Genomic analysis revealed that most tumors spontaneously acquired the Trp53 mutation, in line with the critical role of p53 loss in CS development (Cuevas et al., 2019). This GEM represents a unique model that exhibits a transition from type I to type II EC. POLE encodes a DNA polymerase epsilon, mutated in 7–12% of ECs, and the P286R mutation drives an ultra-mutator phenotype (Kandoth et al., 2013; Rayner et al., 2016). BAC-Sprz2f-Cre; Pole$^{P286R/+}$ mice developed EC with complete penetrance, which exhibited mutation signatures similar to human EC, while remaining relatively stable in gene copy number. These findings are consistent with those of POLE-type ultra-mutated EC. Further tumor progression is achieved by concurrent Msh2 deletion (Li et al., 2020).

### Sprz2f-Cre Mice

Sprz2f is exclusively expressed in the uterus, particularly in endometrial epithelial cells (Conterras et al., 2010). Hence, it may be an ideal candidate for a gene promoter used in Cre transgenic mice for EC development. To this end, Sprz2f-Cre transgenic mice were generated by fusing a 5.8 kb Sprz2f promoter fragment with Cre. Despite uterus-specific expression of endogenous Sprz2f, Cre activity in this mouse was observed not only in the endometrium but also in the kidney and cerebellum (Conterras et al., 2010), as demonstrated by the ROSA26 reporter knock-in mice (R26R) (Soriano, 1999). This ectopic leaky expression in Sprz2f-Cre mice might be attributed to the short length of the promoter fragment, which could lack distant regulatory elements required for the repression of its non-endometrial expression. Furthermore, gene recombination in the adult uterine epithelia exhibited a mosaic pattern. Thus, there might be room for further refinement of these Sprz2f-Cre mice. Nevertheless, Sprz2f-Cre; Lkb1$^{fl/fl}$ mice develop invasive ECs and eventually die by 30 weeks of age (Conterras et al., 2010). Concurrent deletion of cytokine chemokine ligand 2 (Ccl2) significantly attenuates Lkb1 loss-driven tumor progression, revealing the involvement of inflammation in EC development (Peña et al., 2015). The development of type II EC has been associated with telomeric dysfunction (Akbay et al., 2008). Consistent with this notion, the concurrent deletion of Trp53 and Pot1a, which encodes a component of the shelterin complex that stabilizes telomeres, in Sprz2f-Cre; Pot1a$^{fl/fl}$; Trp53$^{fl/fl}$ mice leads to the development of type II EC (Akbay et al., 2013).

### BAC-Sprz2f-Cre Mice

To recapitulate the highly endometrium-specific transcriptional regulation of Sprz2f in mice, the bacterial artificial chromosome (BAC) clone RP23-3914 was introduced to drive Cre expression instead of the shorter promoter sequence used in Sprz2f-Cre mice. Since the BAC clone harbors a 189 kb genomic fragment spanning the Sprz2 tandem gene cluster region on chromosome 3, it is postulated to be long enough to recapitulate all endogenous transcriptional regulation accurately. Indeed, BAC-Sprz2f-Cre transgenic mice allowed gene recombination in a highly endometrium-specific manner. EC has the highest incidence of FBXW7 mutations among all human cancers, followed by colon cancer (Yeh et al., 2018). BAC-Sprz2f-Cre; Pten$^{fl/fl}$; Fbxw7$^{fl/fl}$ mice developed endometrioid carcinoma, which eventually progressed to CS at 40–67 weeks. Genomic analysis revealed that most tumors spontaneously acquired the Trp53 mutation, in line with the critical role of p53 loss in CS development (Cuevas et al., 2019). This GEM represents a unique model that exhibits a transition from type I to type II EC. POLE encodes a DNA polymerase epsilon, mutated in 7–12% of ECs, and the P286R mutation drives an ultra-mutator phenotype (Kandoth et al., 2013; Rayner et al., 2016). BAC-Sprz2f-Cre; Pole$^{P286R/+}$ mice developed EC with complete penetrance, which exhibited mutation signatures similar to human EC, while remaining relatively stable in gene copy number. These findings are consistent with those of POLE-type ultra-mutated EC. Further tumor progression is achieved by concurrent Msh2 deletion (Li et al., 2020).

### Ltf-iCre Mice

Lactoferrin (Ltf), a non-heme iron-binding glycoprotein, is highly expressed in the uterine epithelium of adult mice in response to estrogen exposure (McMaster et al., 1992; Teng et al., 2002). Ltf-improved Cre (iCre) transgenic mice were generated as an endometrium-specific iCre mouse line. However, ectopic expression was observed in mammary glands and neutrophils (Daikoku et al., 2014). iCre is a modified Cre gene that reduces the high CpG content of the prokaryotic coding sequence, thereby reducing the chances of epigenetic silencing in mammals (Shimshek et al., 2002). Whereas Pgr-Cre; Pten$^{fl/fl}$ mice develop EC with high penetration (Daikoku et al., 2008), Ltf-iCre; Pten$^{fl/fl}$ mice develop uterine complex atypical hyperplasia, but not carcinoma (Liang et al., 2018). These results suggest that Pten inactivation in the uterine stroma can promote the transformation of Pten-null epithelial cells, which may not be potently tumorigenic on their own. Considering that Pten inactivation in the uterine stroma is
observed in Cowden’s disease, but not common in sporadic cases of human EC, these results suggest that Pgr-Cre; Ptenfl/fl mice and Lfii-Cre; Ptenfl/fl mice may mimic hereditary and sporadic cases of EC, respectively. ARIDIA loss frequently co-occurs with mutations in genes involved in the PI3K pathway in ECs (Liang et al., 2012). Consistent with this finding, Pgr-Cre; R26-Pik3caH1047Rfl, Arid1afl/+ mice develop ECs (Wilson et al., 2019), suggesting that ARIDIA is a haploinsufficient tumor suppressor in EC development.

**Ksp1.3-Cre Mice**

Ksp-1.3 (also known as Cdh16) is expressed exclusively in the adult kidney and developing genitourinary tract in mice (Thomson et al., 1995; Thomson et al., 1998). Ksp1.3-Cre activity is usually observed in a mosaic pattern in endometrial epithelial cells of the lumen and glands (Shao et al., 2002; Frew et al., 2008). Ksp1.3-Cre; Trp53fl/+ mice develop type II ECs, such as serous carcinoma, clear cell carcinoma, and CS, in 84% of cases at 58–68 weeks of age (Wild et al., 2012). In these tumors, the mTORC1 signaling pathway is frequently activated in precancerous lesions and tumors, suggesting that its cooperation with Trp53 loss leads to the development of type II EC.

**Pax8-rtTA; TetO-Cre Mice**

Pax8 is a lineage-specific transcription factor that marks the Müllerian lineage epithelium, such as the uterus and FT, but not the ovary (Mittag et al., 2007). The Pax8-rtTA; TetO-Cre mouse contains a reverse tetracycline (Tet)-regulated transactivator under the control of the murine Pax8 promoter and TetO-Cre, thereby enabling gene recombination in an inducible manner by doxycycline administration. Using this mouse model, the effects of various combinations of conditional alleles of Brcal1/2, Trp53, and Pten were investigated. While these mice predominantly developed high-grade serous carcinoma from secretory epithelial cells in the FT, various degrees of endometrial lesions were also induced to a lesser extent, resembling endometrial secretory epithelial cells in the FT, various degrees of endometrial hyperplasia and dysplasia, and carcinoma in humans (Perets et al., 2012). ARIDIA loss-dependent tumorigenesis, which Cre expression is induced by tamoxifen administration (Hayashi and McMahon, 2002), intraperitoneal (i.p.) injection of tamoxifen leads to gene recombination mainly in epithelial cells throughout the body in a dose-dependent manner. After i.p. injection of tamoxifen, CAG-Cre-ER2; Ptenfl/+ mice developed endometrial hyperplasia and adenocarcinoma in situ, while thyroid hyperplasia was also ectopically observed (Mirantes et al., 2013). These results underscore the higher susceptibility of the endometrium to Pten loss-dependent tumorigenesis compared to any other organ. However, considering the ubiquitous expression pattern, it remains to be seen whether transgenic CAG-Cre-ER2 mice are also useful in EC development driven by other genetic alterations.

**Amhr2-Cre Mice**

Amhr2 is expressed in the Müllerian duct mesenchyme and the adjacent mesonephric epithelium (Arango et al., 2008). Amhr2-Cre mice undergo gene recombination in the uterus, selectively in the cells of the stroma and myometrium, but not in the epithelium (Jamin et al., 2002; Daikoku et al., 2013). Amhr2-Cre; Ptenfl/+ mice fail to develop EC, but instead potently induced the conversion of myometrial cells into adipocytes (Daikoku et al., 2011). Amhr2-Cre; Ptenfl/+; KrasL52-G12D+/+ mice develop uterine stromal tumors, such as leiomyoma and leiomyosarcoma, but not epithelial tumors (Kun et al., 2020). These findings suggest that Amhr2-Cre mice may not be suitable for uterine epithelial tumorigenesis.

**Intrauterine Injection of Adenovirus-Cre**

As an alternative approach to achieve conditional gene recombination in the endometrium of adult mice, local injection of adenovirus encoding Cre (Adeno-Cre) has been adopted (Beauparlant et al., 2004). After the intrauterine (i.u.) injection of Adeno-Cre, Ptenfl/+ mice developed EC, albeit with partial penetrance (Joshi and Ellenson, 2011; Saito et al., 2011). After Adeno-Cre injection, Ptenfl/+; KrasL52-G12D+/+ and Ptenfl/+; Lkb1fl/+ mice developed EC with 50% penetrance (Tirodkar et al., 2014) and 100% penetrance (Cheng et al., 2014), respectively. These results suggest that Lkb1 inactivation cooperates more profoundly than Kras activation. However, both genetic alterations are thought to activate the PI3K pathway. Visualization of gene recombination using R26R mice demonstrated that gene recombination by the i.u., injection of Adeno-Cre is not completely epithelium-specific or uniformly achieved in targeted cells (Joshi and Ellenson, 2011). Nonetheless, it seems likely that the Adeno-Cre approach has been successful, at least for modeling EC development driven by Pten deletion.

**EX VIVO/IN VIVO HYBRID MOUSE MODELS FOR EC**

**General Overview**

In addition to pursuing tissue specificity in vivo by developing novel Cre transgenic mice with the promoter of endometrium-specific genes, researchers have also attempted to achieve the same goal using an alternative approach. It is a cell-based carcinogenesis
model comprising two steps: 1) \textit{ex vivo} culture of murine primary endometrial cells followed by gene transduction to reconstitute EC-specific mutation and 2) allograft implantation of transduced cells into immunocompromised mice for \textit{in vivo} monitoring of tumor development. An obvious advantage of this \textit{ex vivo/in vivo} hybrid approach is that the cell and tissue type specificity is warranted by physical separation of the uterus and that the outcome is apparent within a short period. On the other hand, the caveat is that the authentic uterine microenvironment, including the proficient immune response, is not thoroughly provided, potentially affecting the outcome depending on the genetic alterations tested in this model. At any rate, no model is perfect by itself. Ideally, they should complement one another to move EC research forward. Below, we have illustrated recent efforts, including ours, to develop cell-based \textit{ex vivo/in vivo} hybrid models for endometrial carcinogenesis.

**Monolayer Cell-Based Model**

To achieve uterine-specific genetic engineering \textit{ex vivo}, the uterus was isolated from wild-type (WT) mice. After enzymatic and mechanical dissociation, primary murine epithelial cells were transiently prepared and expanded in a Petri dish. Genetic engineering, such as the introduction of \textit{myr-Akt} (encoding a membrane-bound constitutive active form of Akt) or sh\textit{Pten} (encoding short hairpin RNA against \textit{Pten}) into primary endometrial cells, is conducted using lentiviral vectors. By implanting transduced endometrial cells, together with the WT uterine stromal cells from neonates, into the renal capsule of severe combined immunodeficiency (SCID) mice, adenocarcinoma development was achieved (Memarzadeh et al., 2010). This study demonstrated that the generation of GEM is dispensable for the induction of EC in murine cells. Similarly, epithelial cells isolated from the uterus of GEM also develop tumors. After isolation of the uterus from \textit{Pten}\textsuperscript{fl/fl} mice, epithelial cells were separated from cultured stromal cells using flow cytometry. After infecting these cells with lentivirus encoding \textit{Cre} (LV-\textit{Cre}), the resultant \textit{Pten}\textsuperscript{null} epithelial cells and WT uterine stroma were jointly implanted into the renal capsule of oophorectomized SCID mice. With estrogen supplementation, adenocarcinoma-like tumors eventually developed (Fanzen et al., 2013). These two studies suggest the importance of cooperation between epithelia and stroma for the development of \textit{Pten} deletion-driven EC, although whether co-transplanted uterine stromal cells are dispensable for tumor development is yet to be investigated. In addition, it remains to be seen whether tumors can develop even in the subcutis, where there is a lower blood supply.

**Organoid-Based Model**

**General Overview**

Organoid culture is an emerging technology that enables the self-renewal and differentiation of normal epithelial stem cells in a matrix-assisted three-dimensional structure supplemented with distinct stem cell niche factors. As organoids are maintained in serum-free media, epithelial cells are selectively propagated in response to EGF, while non-epithelial cells are quickly and automatically depleting from the culture. Thus, flow cytometry-based sorting is not necessary for the purification of epithelial cells. It was first demonstrated that murine intestinal stem cells marked by \textit{Lgr5} could infinitely proliferate \textit{in vitro}, giving rise to all differentiated lineages of the small intestine (Sato et al., 2009). Organoid culture technology has been extensively applied to various cancer research fields, including carcinogenesis, drug resistance, and drug discovery (Drost and Clevers, 2018). We previously developed an efficient \textit{in vitro} gene transduction method using a lentivirus (Maru et al., 2016), which is based on the Matrigel bilayer organoid culture (MBOC) protocol (Maru et al., 2019a). This technical innovation enabled us to establish the first murine organoid-based carcinogenesis model with WT normal cells in the \textit{ex vivo/in vivo} setting (Figure 1). It recapitulated multi-step colon carcinogenesis in the subcutis of nude mice without generating GEM. Similarly, multiple genetic alterations were reconstituted in murine and human intestinal organoids using CRISPR/Cas9 technology to generate organoid models of \textit{multi-step colorectal carcinogenesis} (Drost et al., 2015; O’rourke et al., 2017). Although tumor development from murine organoids is achieved by one or two genetic alterations, it remains technically challenging to induce full-blown tumors from normal human organoids even with three to four genetic alterations. This suggests that murine cells might have a lower threshold for tumorigenesis, presumably reflecting a shorter lifespan in mice.

Using this approach, we demonstrated that reconstitution of the common genetic alterations in each organ could considerably and generally recapitulate multi-step carcinogenesis independently of the tissue-specific microenvironment. For example, mutant \textit{Kras} in intestinal organoids markedly accelerates tumorogenesis caused by \textit{ApC} knockdown alone (Onuma et al., 2013). In organoids from the hepatobiliary tract (Ochiai et al., 2019) and pancreas (Matsuura et al., 2020), full-blown tumors are developed not by mutant \textit{Kras} alone but by concurrent inactivation of the p53 or Rb pathways. These outcomes are consistent with the results from earlier \textit{in vivo} GEM studies with identical genetic alterations, if the tumorigenic potential was evaluated based on the tumor development rate, size, and histology. Therefore, these findings point toward the notion that these models could, at least partly, substitute and complement GEMs in evaluating the pathological relevance of reconstituted genetic interactions. \textit{Ex vivo/in vivo} hybrid carcinogenesis models using murine FT organoids have been recently described (Maru et al., 2021b). Although \textit{Trp53} loss alone is not sufficient for tumor development in FT organoids, the concurrent introduction of common genetic aberrations in ovarian high-grade serous carcinoma leads to the development of tumors of various grades and histological features. For example, adding \textit{Pik3ca}\textsuperscript{H1047R} or \textit{Kras}\textsuperscript{G12D} to \textit{p53}\textsuperscript{null} FT organoids leads to the development of adenocarcinoma and CS, respectively. Drug sensitivity varies among mouse-derived tumor organoids (MDTO) with different genetic aberrations.

Given the ectopic nature as a tumor developing site and the lack of the right immune response, the subcutis of Balb/c background nude mice may not be ideal for \textit{C57bl/6} background endometrial organoids for tumor development. However, we initially did not pay much attention to this situation because the purpose of inoculating transduced organoids in nude mice was to detect cellular transformation, which was supposed to be achieved by genetic engineering alone. Intriguingly, a detailed analysis of MDTO revealed that this artificial setting supported \textit{Kras}-driven carcinogenesis from pancreatic organoids in a way that recapitulates pancreatic carcinogenesis in humans (Matsuura
For example, organoids acquired genetic or epigenetic changes after subcutaneous inoculation, which were, in turn, positively selected by nude mice for more malignant tumors. Moreover, mutation signatures in MDTO were similar to those observed in human pancreatic cancer. Based on these findings, it is tempting to speculate that the hybrid carcinogenesis models might depend on both ex vivo and in vivo parts. Both would further cooperate for tumorigenesis. Thus, we updated our view on ex vivo/in vivo hybrid carcinogenesis models that had initially emphasized the ex vivo part.

**Endometrial Organoid-Based Model**

Murine endometrial organoids recapitulate the physiological response of the endometrial epithelium to hormones, including estrogen and progesterone (Boretto et al., 2017). However, it remains unclear whether carcinogenesis could also be recapitulated. We recently aimed to establish an ex vivo/in vivo hybrid carcinogenesis model using endometrial organoids (Maru et al., 2021a). Unlike in earlier in vivo studies using GEM, inactivation of Pten in endometrial organoids has only a marginal impact on tumorigenesis, even when combined with mutant Kras. These results reconfirmed the relevance of the uterine stroma in endometrial carcinogenesis. We are currently testing many other combinations of genetic alterations that could potentially cooperate for EC development. We have already generated multiple tumors, and detailed information will be reported elsewhere.

Intriguingly, the propagation of endometrial organoids was unexpectedly halted after the lentiviral introduction of the pLKO.1-puro vector and shLuc (shRNA against luciferase), an empty backbone vector and the negative control shRNA, respectively (Maru et al., 2021a). These phenomena have never been observed in any other tissue-derived organoids under almost the same culture conditions (Onuma et al., 2013; Ochiai et al., 2019; Matsuura et al., 2020; Maru et al., 2021b). In addition, endometrial organoids with potentially advantageous alterations, such as Cre-mediated Kras \(^{G12D}\) induction or shRNA-mediated Pten knockdown, continued to proliferate over many passages after lentiviral infection. Together, these observations raise the possibility that endometrial organoids may be extremely sensitive to DNA damage triggered by genome integration following lentiviral infection, which could be overcome by oncogenic signals. Alternatively, this might imply that the currently adopted culture conditions require further optimization for endometrial organoids.

Notably, Cdkn2a knockdown or Trp53 deletion in Kras \(^{G12D}\) expressing endometrial organoids leads to CS or monophasic sarcoma development via extensive epithelial-mesenchymal transition (Maru et al., 2021a). Re-inoculating MDTO into the subcutis of nude mice revealed that the transition from carcinoma to CS might be unidirectional. Considering that identical genetic aberrations in pancreatobiliary organoids always result in the development of adenocarcinoma (Ochiai et al., 2019; Matsuura et al., 2020), these findings, together with the results of a study with FT organoids, suggest that gynecological organs, in general, may be predisposed to CS due to some inherent epigenetic status. Detailed examination of organoids in each step of the models revealed that spontaneous deletion of the Kras \(^{WT}\) allele frequently occurs in MDTOS with Kras \(^{G12D}\) and shCdkn2a knockdown, but not in those with Kras \(^{G12D}\) and Trp53 deletion. These findings are in line with the notion that the Kras \(^{WT}\), which is normally regarded as an oncogene, serves as a relative tumor suppressor gene in the presence of Kras \(^ {G12D}\) (Zhang et al., 2001), suggesting that Cdkn2a suppression might be less tumorigenic than Trp53 loss in the context of Kras-driven tumorigenesis in gynecological organs. It remains to be seen whether tumor development can be achieved in GEM models with identical genetic alterations.

**DISCUSSION**

The development of genetic mouse models for EC has been extended in two different directions. One conventional method is to pursue the generation of GEMs with the highest specificity for genetic engineering of the uterine epithelium. To this end, the
two Cre lines, Ltf-iCre (Daikoku et al., 2014) and BAC-Spr2f-Cre (Cuevas et al., 2019), are currently the most sophisticated in terms of accurate genetic engineering of the endometrium. As the cooperation between epithelial cells and the microenvironment, such as stromal cells and immune cells, is also integrated, these in vivo GEM models will likely contribute to the elucidation of the molecular mechanisms underlying EC initiation and progression. Hence, it is unlikely that there is a growing demand for the development of novel superior Cre lines in generating EC models. Researchers would rather generate more GEMs with diverse combinations of genetic alterations using these available Cre lines. Most previous studies mainly focused on histological features but not genomic, transcriptomic or proteomic features concerning the similarity between induced mouse uterine tumors and human EC. Now that public databases of so-called Omics-based analysis of human tumors have become available, future studies will definitely need to include these data in assessing the inter-species similarity of the tumors.

Another new way is to develop ex vivo/in vivo hybrid models based on genetic engineering of primary endometrial cells or organoids. Given the rapid application of organoids and CRISPR/Cas9 technology in many research fields (Izumiya et al., 2021), it is probable that an increasing number of organoid-based models for EC will be developed. However, only a few such models have been documented for FRT organs to date (Zhang et al., 2019; Lohmusar et al., 2020; Maru et al., 2021a; Maru et al., 2021b), including ours being the first and only EC model (Maru et al., 2021a). Although this study described the development of a novel model for uterine CS, it belongs to a minor category of type II EC, and no organoid-based model for type I EC has been developed so far. However, in an ongoing project, we are developing new organoid-based type I EC models. These results will also shed light on the mechanisms by which the outcomes could vary between in vivo and ex vivo/in vivo hybrid models for EC.

One of the obvious shortcomings of the current ex vivo/in vivo hybrid carcinogenesis model is the absence of a proficient immune response and physiological tissue-specific microenvironment. To overcome this limitation associated with the subcutis of nude mice, we recently established an orthotopic gallbladder (GB) cancer model using syngeneic mice (Kato et al., 2021). Specifically, we generated genetically engineered organoids in vitro using LV-Cre and CRISPR/Cas9 technology. The organoids were inoculated into the dorsal skin of a syngeneic WT mouse, which developed subcutaneous tumors in several weeks. Subsequently, minced tumor fragments were directly sutured to the outer surface of the GB of another syngeneic WT mouse. This novel two-step model enabled detailed analysis of tumor-infiltrating immune cells and evaluation of drug responses in more physiological settings. Notably, the therapeutic effects of immune checkpoint inhibitors will become feasible.

In our earlier studies, the subcutis of nude mice was selected as the target site for organoid inoculation. Still, it was mainly for technical convenience and did not necessarily mean using nude mice is required. Indeed, we confirmed that transduced pancreatic organoids could develop in the subcutis of syngeneic mice, albeit smaller in size (Matsuura et al., 2020). Moreover, orthotopic implantation of or tumor organoids or fragments derived from nude mice efficiently developed tumors in the pancreas. Similarly, we obtained subcutaneous tumors in syngeneic mice with a subset of endometrial organoids expressing KrasG12D and shRNAs targeting certain tumor suppressor genes. Therefore, the application of the abovementioned two-step approach to transduced endometrial organoids that are proven tumorigenic in nude mice might be worth testing and should be further pursued. Such efforts will probably lead to significant improvements in the current organoid-based EC model as a preclinical model.

CONCLUDING REMARKS

Mouse genetic models that recapitulate EC development to varying extents in in vivo or ex vivo/in vivo hybrid settings have been developed. Precise modeling of EC and mutual comparison will contribute to both basic and translational research on EC. We hope that this review article will help researchers in the field to develop novel EC models.

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