Fbxw7 is a driver of uterine carcinosarcoma by promoting epithelial-mesenchymal transition

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Uterine carcinosarcoma is an aggressive variant of endometrial carcinoma characterized by unusual histologic features including discrete malignant epithelial and mesenchymal components (carcinoma and sarcoma). Recent studies have confirmed a monoclonal origin, and comprehensive genomic characterizations have identified mutations such as Tp53 and Pten. However, the biological origins and specific combination of driver events underpinning uterine carcinosarcoma have remained mysterious. Here, we explored the role of the tumor suppressor Fbxw7 in endometrial cancer through defined genetic model systems. Inactivation of Fbxw7 and Pten resulted in the formation of precancerous lesions (endometrioid intraepithelial neoplasia) and well-differentiated endometrioid adenocarcinomas. Surprisingly, all adenocarcinomas eventually developed into definitive uterine carcinosarcomas with carcinomatous and sarcomatous elements including heterologous differentiation, yielding a faithful genetically engineered model of this cancer type. Genomic analysis showed that most tumors spontaneously acquired Tp53 mutations, pointing to a triad of pathways (p53, PI3K, and Fbxw7) as the critical combination underpinning uterine carcinosarcoma, and to Fbxw7 as a key driver of this enigmatic endometrial cancer type. Lineage tracing provided formal genetic proof that the uterine carcinosarcoma cell of origin is an endometrial epithelial cell that subsequently undergoes a prominent epithelial–mesenchymal transition underlying the attainment of a highly invasive phenotype specifically driven by Fbxw7.

uterine carcinosarcoma | Pten | Fbxw7 | epithelial-mesenchymal transition | Tp53

Endometrial carcinoma (EC), which arises in the endometrial lining of the uterine corpus, is a common malignancy in women, with over 60,000 cases anticipated in the United States this year (1). Most cases are well-differentiated and of the endometrioid subtype, where all of the malignant cells are epithelial and form glands resembling those of normal endometrium. Such cancers are usually confined to the uterus at the time of diagnosis, and, for such tumors, the prognosis is good. However, the other principal EC histologic subtypes—serous carcinoma, clear cell carcinoma, and carcinosarcoma—are of higher histologic grade and have a much worse prognosis (2). Among these, uterine carcinosarcoma (UCS; previously known as malignant mixed Müllerian tumor [MMMT]) is by far the most lethal, with a tendency for early and widespread metastases and an estimated 5-y survival of only 30%. UCS accounts for only 3% of endometrial cancers, but 16% of deaths (3).

UCS is an intriguing EC subtype defined by “biphasic” histology consisting of admixed malignant epithelial and mesenchymal (i.e., carcinomatous and sarcomatous) components (2). The epithelial component is low or high grade and resembles either endometrioid or serous adenocarcinoma (4). The mesenchymal component can be “homologous” with types of differentiation native to the uterus, such as smooth muscle or endometrial stroma. Remarkably, however, the mesenchymal component is conspicuously “heterologous” in half of cases, exhibiting, for example, obvious cartilaginous or osseous differentiation (chondrosarcoma or osteosarcoma) (5).

Historically, UCS was considered a sarcoma, but recent studies have argued it represents a variant of uterine carcinoma. For example, UCS shares epidemiologic features and patterns of chromosomal instability with high-grade carcinomas, and also shares mutational spectra with ECs, including frequent mutations in loci encoding PI3K pathway components (6–8). Comparison of

Significance

Uterine carcinosarcoma (UCS) is an aggressive endometrial cancer variant distinguished from endometrial adenocarcinoma (EC) by admixed malignant epithelial and mesenchymal components (carcinoma and sarcoma). The molecular events underlying UCS are enigmatic, as cancer gene mutations are generally shared among UCS/EC. We take advantage of genetic approaches in mice to show that inactivation of Fbxw7 and Pten results in UCS through spontaneous acquisition of mutations in a third gene (Tp53), arguing for strong biological selection and synergism in UCS. We used this UCS model including tumor-derived cell lines to show that Fbxw7 loss drives epithelial–mesenchymal transition, explaining Fbxw7’s role in UCS. This model system argues that simultaneous genetic defects in 3 distinct pathways (Fbxw7, Pten/PI3K, Tp53) converge in UCS genesis.

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microdissected epithelial and mesenchymal components from individual patients has revealed common chromosomal alterations and mutations, arguing for a monoclonal origin (9–11). Systematic genomic characterizations of UCs have identified frequent mutations in some genes, most notably Tp53, but such mutations also frequently occur in serous and high-grade endometrioid cancers and are not specific to UCS (6). These findings, taken together, thus beg the question as to the nature of the specific molecular driver(s) of EMT/sarcomatous differentiation in UCS. Also, whether the UCS cell of origin is epithelial or mesenchymal (and whether the presence of the 2 components represents an epithelial–mesenchymal transition or conversely a mesenchymal–epithelial transition) cannot be definitively ascertained from human studies. No definitive precursor lesion has been described for UCS, adding further uncertainty as to its histogenesis.

Fbxw7 (previously known as CDCA4) is a tumor suppressor that functions as the substrate recognition component of an E3 SCF-type ubiquitin ligase complex controlling the degradation of key cell growth regulators including c-Myc, Cyclin E, Notch1, Kii5, and mTOR (12–14). Fbxw7 targets are regulated in a tissue-specific and/or developmental manner (15, 16). Fbxw7 mutations characterize diverse cancers (hematopoietic, colon, stomach, gallbladder/bladder duct). In women, carcinomas of the lower female reproductive tract (the Müllerian system), including the uterine cervix and corpus, frequently harbor Fbxw7 mutations. Indeed, uterine cancers have the highest incidence of Fbxw7 mutations among all human cancers, followed by colon cancer (17). Although many Fbxw7 mutations are heterozygous and include missense mutations in the substrate binding or WD40 domain, a significant number of mutations are truncating or otherwise loss-of-function. This spectrum of Fbxw7 mutations appears to have similar biochemical consequences, resulting in the inactivation of Fbxw7 and stabilization of Fbxw7 cell type-specific substrates. Concordantly, in diverse nongynecologic mouse models, both the WD40 and truncating mutations have proven potently oncogenic (14, 18–20).

Notwithstanding the importance of Fbxw7 in EC as evidenced by its high mutation rate (10 to 20%) (17, 21), little is known about its biological functions as an endometrial tumor suppressor. Despite numerous studies of Fbxw7 in other cancer types in mice, no Fbxw7-based mouse models of EC have been generated to date. Here, we describe an endometrial-specific Cre driver and used it to investigate the role of Fbxw7 as an endometrial tumor suppressor through concerted genetic analyses in mice, complemented with investigations of human uterine cancer samples. These investigations revealed an unexpected role of Fbxw7 as a driver of EMT in the genesis of UCS.

**Results**

**BAC-Sprr2f-Cre, an Improved Endometrial Cre Driver for Genetically Engineered EC Models.** We previously showed that the murine Sprr2f gene is expressed only in the uterus, and therein only within endometrial epithelium, the presumptive cell of origin for all ECs. In these prior studies, a 5.5-kb Sprr2f promoter fragment was fused to Cre to generate a “minigene” transgenic driver line Sprr2f-Cre, a.k.a. Tg(Sprr2f-Cre)1Dm (22). This allele drives Cre-mediated recombination in endometrial epithelium and has been used in the generation of EC models (23–25). However, one limitation is ecotropic Cre expression, including in kidney and cerebellum. Since the endogenous Sprr2f gene is expressed only in the uterus and not in these other tissues, we surmised that undesired ectopic activity was due to the small promoter fragment and absence of distant regulatory elements repressing nonendometrial expression (22).

To overcome these limitations, we took advantage of bacterial artificial chromosome (BAC) RP23-3914A (26), which harbors a 189-kb C57/B6 genomic fragment spanning the Sprr2 tandem gene cluster on chromosome 3. Recombining methods were used to replace the Sprr2f coding sequence with a Cre ORF at the ATG start codon in exon 2 (Fig. 1A). TheNeo selection cassette was then excised by transient flippase expression, and the resulting engineered BAC was injected into mouse oocytes for generation of a transgenic BAC-Sprr2f-Cre founder line. In BAC transgenesis, integration occurs at random chromosomal locations. The site of integration for BAC-Sprr2f-Cre was not determined, but is autosomal based on its Mendelian pattern of inheritance.

Crossto the Rosa26 β-galactosidase reporter (R26R) (27) were conducted to compare patterns of Cre-mediated recombination of the original Sprr2f-Cre versus the BAC-Sprr2f-Cre line. No Cre activity was observed with R26R alone, as expected. With the original Sprr2f-Cre allele, extensive Cre-mediated recombination was observed in the uterus (endometrial epithelium), kidney (tubular epithelium), and cerebellum (Purkinje cells), as previously reported. In contrast, with the new BAC allele, ectopic expression in cerebellum and kidney was abolished, while recombination efficiency in endometrium was unaltered (Fig. 1B). Ectopic expression in fallopian tube epithelium was also abolished (Fig. 1C). In BAC-Sprr2f-Cre;R26R uteri, endometrial epithelium exhibited ∼50% recombination by 6 wk of age. This recombination efficiency (i.e., subtotal, mosaic) is useful for genetically engineered mouse models of EC, as it recapitulates the admixture of genetically normal and abnormal epithelial cells that characterize the initial stages of endometrial carcinogenesis (28). Cre-mediated recombination was not observed in sexually immature BAC-Sprr2f-Cre;R26R mice at 3 wk of age, but was efficiently induced by β-estradiol (Fig. 1D). This confirms that the estrogen dependence of the Sprr2f locus (which harbors estrogen response elements) is retained with BAC-Sprr2f-Cre and also that Cre-mediated recombination can be induced early by β-estradiol administration (22).

**Fbxw7 and Pten Potently Synergize to Drive ECs in Mice.** Fbxw7 mutations in human EC were first reported in 2002 (29), but their significance and high incidence (∼20% of cases) was not fully appreciated until genome sequencing efforts confirmed and extended the initial results (21). Despite the crucial contribution of Fbxw7 mutation in EC, few studies have focused on the biological basis of its function as an endometrial tumor suppressor in vivo. To develop an animal model to explore these critical questions, we first studied Fbxw7 inactivation alone. BAC-Sprr2f-Cre was bred to mice harboring a floxed Fbxw7 allele, where LoxP sites flank essential exons 5 to 6 (14). The absence of tumors up to 1 y of age in BAC-Sprr2f-Cre;Fbxw7fl/fl females (abbreviated Fbxw7) implied that Fbxw7 inactivation alone was insufficient to drive ECs (Fig. 2A) and that other cooperating genetic events contribute. This is also consistent with observations that ECs with Fbxw7 mutations harbor multiple oncogenic mutations. Several observations pointed to Pten inactivation as a potential cooperating genetic event. First, the PI3K pathway is frequently dysregulated in EC, and Pten, a potent inhibitor of PI3K signaling, is the most frequently mutated gene in EC (30). Analyzing the Uterine Corpus EC TCGA data through cBioPortal (31), we found that, of 43 Fbxw7 mutant cases, all but 8 had mutations in canonical PI3K pathway genes. Furthermore, Pten was the PI3K pathway gene mutated in the highest percentage of cases (25/43, 58%), followed by Pik3ca (20/43, 47%).

To investigate cooperation between Fbxw7 and Pten in endometrial carcinogenesis, cohorts of double mutant BAC-Sprr2f-Cre:Fbxw7fl/fl;Ptenfl/fl (abbreviated Fbxw7/Pten) were established for longitudinal studies including survival analysis. Cohorts of single gene knockout Fbxw7 or Pten mice were also generated. In contrast to Fbxw7-alone mice, Fbxw7/Pten mice developed uterine cancers starting at 12 wk of age. The tumors were bulky but localized, typically involving only 1 of the 2 uterine horns (Fig. 2B). Clonal losses of Pten protein were confirmed immunohistochemically. By 6 wk, Pten was lost in ∼50% of epithelial cells, and these clones also showed increased p-Akt(Ser473), consistent
with Akt hyperactivation. This led to complete relocalization of the transcription factor and Akt target Foxo1 from the nucleus to the cytoplasm, as occurs in human endometrial precancers and other PI3K-dependent developmental and reproductive processes (32–34). By 12 wk, Fbxw7/Pten mice showed increased percentage of Pten-null cells relative to Pten alone (P = 0.027, t test), showing that Fbxw7 and Pten loss act synergistically in conferring a growth advantage to endometrial epithelial cells (SI Appendix, Fig. S1A and B).

Uterine weights and histology confirmed striking cooperativity between the 2 tumor suppressors. Fbxw7 mice exhibited only small increases in uterine weight, and, while Pten mice showed larger increases, this was due to florid endometrial hyperplasia (a.k.a. endometrioid intraepithelial neoplasia [EIN]) without invasion (Fig. 2C). By 24 wk of age, the difference in uterine weights between Fbxw7/Pten vs. control mice was statistically significant (P < 0.0025, t test), and this difference became even more significant at 36 wk of age (P < 0.0001, t test). Furthermore, 75% of Fbxw7/Pten mice exhibited myometrial invasion by 24 wk, vs. 0% for Pten alone (P = 0.007, Fisher’s exact test). Per log-rank analysis, survival of Fbxw7/Pten mice was significantly decreased vs. Fbxw7, Pten, or control mice (P < 0.0001 for each of the pairwise comparisons). Fbxw7 mice showed slightly but significantly increased survival vs. Pten alone (P = 0.0151; Fig. 2D).

**Fig. 1.** Generation of an endometrial-specific Cre driver. (A) BAC targeting strategy to generate BAC-Spr2f-Cre construct and transgenic mice. Direction of transcription is shown for all Spr2 genes in BAC RP23-3914 189 kb genomic fragment based on end-sequencing of insert. The Spr2f ORF is entirely encoded within exon 2. FRT, sites for flippase recombination. (B) Evaluation of Cre-mediated recombination in mice harboring R26R β-galactosidase reporter only, Spr2f-Cre;R26R, and BAC-Spr2f-Cre;R26R. Tissues were stained with X-gal: gross (Left) and histological (Right) analysis of slides counterstained with H&E. C, cerebellum, (Scale bars: gross photographs, 2 mm; photomicrographs, 20 μm.) (C) Analysis of Müllerian structures including uterus ("U"), fallopian tube (FT), and ovary ("O"). With BAC-Spr2f-Cre, ectopic expression in FT was eliminated. (D) Impact of treatment with 17β-estradiol (E2) at 3 wk of age. E2 diffusely induced recombination in endometrial epithelial cells. Sections counterstained with H&E. (Scale bars: gross photographs, 2 mm; photomicrographs, 45 μm.)

**Fig. 2.** Uterine Invasive Cancers Are Initially Endometrioid Adenocarcinomas that Unexpectedly Progress to Carcinosarcoma. To investigate histologic progression and behavior of cancers in this model, Fbxw7/Pten mice were necropsied at 6, 12, 24, and 36 wk (n = 6 to 10 per time point; Fig. 3). By only 6 wk of age, 90% of mice exhibited striking endometrioid intraepithelial neoplasia (EIN), the histologic precursor for endometrioid adenocarcinoma (2). EIN lesions were characterized by hypercellularity, glandular complexity, and absence of invasion, closely mimicking human EIN (Fig. 3B). While it is difficult to reliably identify invasion within endometrium (i.e., into endometrial stroma) in humans or mice, the presence of malignant glands/epithelium in myometrium is a definite indicator of invasion in mouse models of EC (22, 35, 36). By 12 wk, 55% of uteri exhibited myometrial invasion, with some exhibiting full-thickness invasion (Fig. 3F). All invasive cancers at 12 wk of age were adenocarcinomas with endometrioid histology and were of an entirely epithelial character. Notably, 50% of these cancers exhibited p63+ squamous differentiation by 24 wk of age, a distinctive hallmark of human EC seen in ≥25% of cases (Fig. 3B) (37). By 36 wk, 80% of the mice harbored invasive ECs.

Histological analyses at 40 to 67 wk revealed a striking, systematic, and unexpected shift of overall tumor histotype. By 67 wk (n = 30), all cancers became biphasic, with distinct but admixed epithelial (carcinomatous) and mesenchymal (sarcomatous) components. The epithelial components consisted of endometrioid adenocarcinoma sometimes with admixed squamous carcinoma. The mesenchymal components consisted of high-grade spindle cells with high mitotic index and high-grade nuclear atypia (undifferentiated sarcoma). Even more strikingly, 100% of these tumors also contained malignant heterologous elements with cartilaginous or osseous differentiation, i.e., chondrosarcoma or osteosarcoma.
Based on reported among prior mouse models of EC, many of which were into definitive UCS. This is remarkable, as UCS has not been shown to metastasize. Tumors begin as usual-type endometrioid adenocarcinomas, but showed metastasis to colon ($27\%$), kidney ($20\%$), and spleen ($13\%$), and $4/15$ mice with highest frequency of metastasis were liver ($53\%$), lung ($40\%$), and cervix, while $58\%$ ($15/26$) showed distant metastasis. The organs survived analysis had metastasis to adjacent organs (ovary, peritoneum), but all mice harbored distant metastases to $1$ site, and these were carcinomatous, sarcomatous, or both. All Fbxw7/Pten−/− (Cre negative, $n=21$), BAC-Spr2f-Cre;Pten−/− ($n=23$), BAC-Spr2f-Cre;Fbxw7−/− (Pten−/−) ($n=30$), and BAC-Spr2f-Cre;Fbxw7−/−;Pten−/− ($n=27$). The Fbxw7/Pten vs. control, Fbxw7/Pten vs. Pten, and Fbxw7/Pten vs. Fbxw7−/+ curves are statistically different ($P < 0.0001$, log-rank test).

We then performed lineage tracing in this UCS model with the mTmG reporter (38) that, upon Cre-mediated recombination, switches from membrane Tomato (red) to membrane green fluorescent protein expression, providing a lineage marker stably inherited over subsequent cell divisions. In mTmG;BAC-Spr2f-Cre females, Cre activity was observed only in endometrial epithelium, with no recombination in endometrial stroma (only endometrial glands turned green). In older females harboring UCS ($n=3$), the sarcomatous and carcinomatous components expressed only green fluorescent protein. This lineage tracing provides formal proof that UCS, including any sarcomatous elements, is ultimately derived from the endometrium (SI Appendix, Fig. S2 A and B).

Concordant with the aggressive behavior of human UCS, most mice harbored distant metastases to $1$ site, and these were carcinomatous, sarcomatous, or both. All Fbxw7−/− mice in the survival analysis had metastasis to adjacent organs (ovary, peritoneum), while $58\%$ ($15/26$) showed distant metastasis. The organs with highest frequency of metastasis were liver ($53\%$), lung ($40\%$), colon ($27\%$), kidney ($20\%$), and spleen ($13\%$), and $4/15$ mice showed metastasis to $2$ organs. We conclude that Fbxw7−/− tumors begin as usual-type endometrioid adenocarcinomas, but evolve in a surprisingly stereotypical manner between 12 and 48 wk into definitive UCS. This is remarkable, as UCS has not been reported among prior mouse models of EC, many of which were based on Pten or Trp53−/− (35, 39–41). These findings thus point to Fbxw7 as a specific driver of UCS. Fbxw7−/− mice represent a faithful mouse model of human UCS, with $100\%$ penetrance for this histotype. Since Cre was expressed solely in the epithelial compartment, the model, together with the mTmG lineage tracing, provides formal genetic proof that the UCS cell of origin is an endometrial epithelial cell that subsequently undergoes EMT.

Unbiased Analyses Reveal that Trp53 Is the Critical Third Cooperating Oncogene in Fbxw7−/−-Driven Murine UCS. The fociality of cancers in Fbxw7−/− uteri (Fig. 2B) strongly suggested that additional, presumably stochastic genetic event(s) drove tumor formation. DNA from 7 formalin-fixed, paraffin-embedded (FFPE) tumors from mice at 28 to 61 wk of age and paired normal samples such as liver were subjected to gene resequencing using a custom hybrid capture panel of 76 murine genes, including all loci corresponding to those frequently mutated in human ECs of all histotypes (SI Appendix, Table S1). DNA obtained from normal tissue served as a matched germline control for reliable identification of somatically acquired mutations (Fig. 4A) (42).

Of the 76 genes, only one—Trp53, the murine homolog of human TP53—was mutated in $\geq 1$ tumor (Fig. 4B). All 4 of these tumors harbored mutations leading to amino acid substitutions (A135V, H165R, V170M, R172H) previously documented as recurring cancer drivers in human tumors (43). For example, murine R172H corresponds to the human R175H dominant-negative hotspot mutation, and an engineered R172H allele also functions as a genetically dominant oncogene in mice (44–46), while murine A135V corresponds to the temperature-sensitive A135V TP53 mutation (47). Only one other mutation was identified among the 76 loci in the 7 cases: KasG12D in a tumor with no Trp53 mutation but with a “mutant pattern” of p53 overexpression (as detailed later and in Fig. 4B).

p53 mutations lead to abnormal accumulation of the protein, making immunohistochemistry (IHC) an extremely reliable surrogate for mutations (48). Whereas control endometria did not express p53 by IHC, 6 of the 7 tumors subjected to sequencing displayed marked overexpression of p53, including 3 of 4 tumors without mutations by sequence analysis (Fig. 4B and C). These results demonstrate that p53 is inactivated in a substantial majority of Fbxw7−/− ECs. Intragenic or larger deletions spanning TP53/Trp53 are common cancer-driving events not detectable by exon resequencing, and may account for the observed overexpression/presumptive mutation of p53 protein in the UCS not harboring Trp53 missense mutations (48).
Fig. 3. Cancer initiation and progression to UCS in Fbxw7/Pten mice. (A) Summary of progression per histologic analyses. (B, Top) Normal endometrium at 12 wk and early noninvasive endometrioid intraepithelial neoplasia (EIN) with preneoplastic architectural features (cell and gland crowding) starting at 6 wk. EIN lesions develop into invasive endometrioid adenocarcinomas (ACA) starting at 12 wk with definitive invasion of malignant glands into myometrium. Some cases exhibit squamous differentiation (sq) confirmed by p63 immunostaining (Inset). (B, Middle) UCS with overtly malignant mesenchymal and epithelial components. The epithelial components are glandular or squamous (sq), whereas mesenchymal components consist of malignant spindle cells with frequent and aberrant mitotic figures (small dashed circles and insets) and foci of malignant cartilage (chondrosarcoma) or bone (osteosarcoma). (B, Bottom) Metastases to peritoneum and peritoneal organs such as ovary and more distant metastasis. Metastatic foci were carcinomatous or carcinosarcomatous. Metastatic deposits highlighted in some panels with dashed white lines. (Scale bars: 50 μm, except where noted.)

To expand upon these results, p53 IHC was performed on a larger set of Fbxw7/Pten tumors. We killed randomly selected mice at 12, 24, and 36 wk (n = 8, 8, and 11, respectively), and also analyzed 27 mice that were subjected to necropsy as part of formal survival analysis (at 39 to 67 wk). Consistent with the aforementioned results, p53 overexpression was not detected in mouse uteri at 12 wk in normal or tumor endometrium. However, the incidence of p53 clonal overexpression by IHC increased over time, reaching 80% in mice euthanized due to illness per tumor burden criteria (Fig. 4 D and E). Of note, some human tumors (those with truncating mutations) exhibit complete loss of p53 expression (so-called null pattern) (48), but this pattern would not be readily detectable due to low levels of p53 protein expression in normal mouse uterine cells, hampering the ability to reliably detect p53 loss relative to adjacent normal cells. Thus, the incidence of p53 inactivation in these tumors may be >80%. Three of the 4 cases (75%) harboring Tp53 mutations exhibited metastases, vs. 4 of 6 (67%) with either Tp53 mutation or mutant-pattern expression p53 and 22 of 27 (78%) among all mice analyzed as part of the survival curve. These differences are not statistically significant. Interestingly, p53 overexpression was detected in many cases by the invasive adenocarcinoma stage (before appearance of sarcoma). For example, at 24 wk, when no tumors exhibited overt UCS features, 40% of tumors already harbored distinctive p53-mutant clones (Fig. 4E). In UCS in older animals, p53 overexpression was observed in both epithelial and mesenchymal components. p53 overexpression (and, by inference, mutation) did not exhibit 1:1 correlation with areas of UCS, either temporally or spatially. These results are similar to human UCS, which typically shows aberrant p53 expression but often only in focal areas, demonstrating that TP53 mutation is a late event (4). Thus, while combined Fbxw7/Pten/Trp53 inactivation appear to be obligate synergistic events in the evolution of UCS, the combination leads to cellular differentiation events not manifest immediately in Trp53-null clones. These findings also suggest that the precursor lesion for UCS can be a preexisting endometrioid adenocarcinoma harboring this mutational triad.

Expression Profiling Reveals a Critical Role for Fbxw7 in Mediating EMT in Uterine Cancers. Cell lines were established from Fbxw7/ Pten UCS at 49 and 56 wk of age (UCS1 and UCS2). To devise an inducible system to study Fbxw7’s biological functions in UCS, a wild-type Fbxw7 cDNA was cloned into a doxycycline-inducible “tet-on” lentiviral construct (pLVX-Fbxw7). Induction of doxycycline resulted in only a modest increase of Fbxw7 protein by Western analysis. We surmised that the modest induction of protein was due to mRNA features such as suboptimal codon utilization limiting protein translation, and synthesized an Fbxw7 cDNA sequence optimized for expression in mammalian cells. The codon-optimized cDNA was cloned into the same lentiviral vector, and the resulting construct (pLVX-Fbxw7opt) resulted in higher Fbxw7 protein levels (SI Appendix, Fig. S3A). UCS1 and UCS2 were transduced with pLVX-Fbxw7opt and subjected to drug selection. Following addition of doxycycline, efficient and concentration-dependent induction of Fbxw7 was documented for both cell lines. As expected, Akt was hyperphosphorylated in UCS1 and UCS2 due to the deficiency of Pten, and induction of Fbxw7 did not have a major impact on Akt phosphorylation levels (SI Appendix, Fig. S3 B and C).

Fbxw7 induction destabilized several canonical Fbxw7 target proteins, including c-Myc, Notch-1 intracellular domain (NICD Val1744), and Krüppel-like factor 5 (Klf5; Fig. 5A), demonstrating that these known Fbxw7 targets likely mediate the actions of Fbxw7 mutations in uterine cancer (14, 20, 49–51). To gain
broader insights into Fbxw7’s functions, UCS1 and UCS2 cells were again infected with pLVX-Fbxw7 lentivirus (+/-), treated with doxycycline, and subjected to transcriptomic profiling by RNA-seq of 3 separately induced biological replicates generated by splitting into 3 separate cell cultures (12 samples total) (S2). Fbxw7 induction exerted a dramatic and reproducible impact on the transcriptome. Using stringent criteria (FDR < 0.001, log
cpm > 0), expression levels of 295 and 59 genes were altered in
UCS1 and UCS2, respectively (Fig. 5B). Of note, the UCS1/2 gene
sets showed significant overlap (48 genes; P value = 1.5e−78; Mate-
rials and Methods). Heat map profiling of the overlapping genes
at FDR < 0.001 confirmed that gene-expression alterations were
uniform among biological replicates in UCS1/2 (Fig. 5C). Gene
Ontology analyses for the larger UCS1 gene set revealed signifi-
cant enrichment for cell adhesion, cell migration, extracellular
matrix organization, Notch signaling, cell matrix adhesion, and
positive regulation of EMT, among other GO terms (Fig. 5D and
SI Appendix, Table S2 A–C). Similar results were obtained for
UCS2 (SI Appendix, Fig. S4 and Table S2 D–F). The differentially
expressed genes include drivers of EMT such as Notch1, Hey li-
gand, Met, Tgfβ1, Pdgfra/b, and extracellular matrix factors be-
lieved to be downstream participants in EMT, such as Mmp2,
Adamts14, and multiple genes encoding collagen and osteoid/
chondroid matrix factors. The differentially expressed GO cate-
gories, genes within each GO category, and differentially expressed
genes are tabulated for UCS1/2 in SI Appendix, Table S2
A–F. Overall, these results point to Fbxw7 as a potent cell-reprogramming
factor and specific driver of EMT in UCS.

To validate these studies, selected differentially expressed genes
were subjected to Western or RT-PCR analysis. Pdgfrβ and col-
lagen 15α1 protein levels were lower following Fbxw7 induction,
concordant with RNA-seq (Fig. 5E). In addition, several other
EMT regulators, including Zeb-1, Twist, β-catenin, and Snail were
also down-regulated following Fbxw7 reexpression (Fig. 5E).
However, some EMT-related proteins such as N-cadherin or Slug were not appreciably altered (Fig. 5E). Leaky expression of the codon-optimized Fbxw7 prior to induction was sufficient to at least partially destabilize targets, with further decreases in protein levels following induction (e.g., c-Myc). We also confirmed differential RNA expression of other genes including HeyL (Notch signaling), Fibulin 5/7 (cell adhesion and extracellular matrix organization), Olfml2a (extracellular matrix organization), Mmp2 (angiogenesis, positive regulation of cell migration), and Mgp (ossification; SI Appendix, Fig. S5).

Evidence for Functional Synergism between PI3K Pathway and Fbxw7 via Gsk3β. Efficient binding of Fbxw7 to target proteins (and hence their degradation) depends on phosphorylation of the degron motif by Gsk3β, creating a phospho-degron. Gsk3β in turn is directly regulated by Akt. Akt phosphorylates Gsk3β, which results in decreased Gsk3β activity and decreased phosphorylation of target degrons (13, 17, 53). To document such potential interactions in this UCS model, we analyzed Gsk3β in the UCS1/2 cell lines. Gsk3β was highly expressed in both lines, and total Gsk3β levels were slightly lower following Fbxw7 reexpression (SI Appendix, Fig. S6A). Treatment with the PI3K inhibitor LY294002 greatly inhibited Gsk3β(Ser9) phosphorylation, confirming that Gsk3β is constitutively phosphorylated by Akt in UCS1/2. This result argues that observed genetic synergism between Fbxw7 and PI3K/Pten pathways is likely to be mediated at least in part by Gsk3β. Concordantly, c-Myc levels were reduced by Fbxw7 reexpression, and this was further enhanced by PI3K inhibition.

In cell growth assays with UCS1/2, Fbxw7 reexpression or PI3K inhibition caused decreased cell growth, but both together exerted a potent synergistic effect (SI Appendix, Fig. S6B). In conclusion, these results argue that the Fbxw7 and PI3K/Pten pathways synergize in UCS through Gsk3β through effects on the stability of Fbxw7 targets such as c-Myc, as previously shown in diverse contexts (13, 17, 53).

Functional Studies Implicate Fbxw7 in Cell Migration and Invasion in Uterine Carinosarcoma. We then studied the role of Fbxw7 in cellular phenotypes related to cancer initiation and progression. Reexpression of Fbxw7 in UCS1/2 showed a consistent, albeit modest, impact on cell proliferation (Fig. 6A), consistent with prior studies showing that Fbxw7 loss promotes cell proliferation through c-Myc and Cyclin E (17). This was also consistent with the growth advantage of Fbxw7/Pten vs. Pten cells documented in vivo (SI Appendix, Fig. S1B). Fbxw7 reexpression impacted cell motility and wound closure in a 2D cell culture system (Fig. 6B) as well as chemotaxis and migration through Matrigel in a Transwell migration assay (Fig. 6C). Moreover, in a separate organotypic experimental culture system, Fbxw7 restricted the ability of cells to extend “invasive” cellular protrusions into the extracellular matrix (Fig. 6D). We then enforced expression of the degradation-resistant mutant c-MycT58A. Gsk3β phosphorylates c-Myc at T58,
and the T58A mutation renders the c-Myc protein insensitive to Gsk3β recognition and Fbxw7-mediated degradation (54). As already shown, endogenous c-Myc is degraded following Fbxw7 reexpression, while c-MycT58A proved resistant (Fig. 6E). c-MycT58A partially reversed the antiproliferative effect of Fbxw7 reexpression, demonstrating that c-Myc is one effector of the observed Fbxw7 phenotypes (Fig. 6E).

We then assessed the properties of 3D organoids derived from Pten, Fbxw7, or Fbxw7/Pten adult uteri not harboring overt tumors and grown in defined media (55, 56). Pten endometrial organoids were significantly larger than controls, but maintained spherical shape, a distinct epithelial monolayer, and hollow interior, giving them a characteristic “donut” morphology. Fbxw7 organoids were also enlarged relative to wt controls, but exhibited a strikingly irregular and ruffled boundary of cells, a phenotype associated with loss of cell adhesion and propensity for invasion. Finally, Fbxw7/Pten organoids exhibited yet another distinct morphology, characterized by very large “cystic” organoids. Morphology and cell boundaries were more clearly delineated by cytokeratin immunofluorescence. Fbxw7 organoids showed absence of a lumen consistent with an EMT phenotype, with loss of cell polarity demonstrated by the apical marker GM130. Cells in Fbxw7/Pten organoids demonstrated increased proliferation compared to their wt counterparts (SI Appendix, Fig. S7). These overall results are consistent with a role for Pten in driving cell proliferation and a synergistic role of Fbxw7 in cell growth and in additional cellular phenotypes associated with EMT and invasive growth. This further rationalizes the observed potent cooperation among Pten and Fbxw7 in tumor initiation and progression.

Evidence for a Role of Fbxw7 in the UCS Phenotype in Humans. Fbxw7 mutations have been identified across several human EC histotypes, including endometrioid and serous adenocarcinomas, and also in UCS. These earlier findings suggested that Fbxw7 mutations occur in UCS but did not ascribe a particular significance to them. To further explore this question, we analyzed available TCGA datasets for uterine adenocarcinomas (including endometrioid and serous subtypes) and UCS (6, 21). Tp53 and Fbxw7 mutations were much more likely to coexist in UCS vs. EC. Mutation incidences were compared for the genes most frequently mutated in UCS and plotted as the inverse of the P value of the difference between the 2 categories (EC vs. UCS, Fisher’s exact test). Importantly, although several genes showed significant differences in mutation frequencies, among these, Tp53 and Fbxw7 were the only genes more frequently mutated in UCS (SI Appendix, Fig. S8A and B). This supports our finding that Tp53 and Fbxw7 mutations synergize in the formation of UCS.
IHC showed that this transition. While Kras and Pten mutations (WD40 hotspot or definite null mutations) and 20 cases without Fbxw7 single nucleotide variants leading to amino acid alterations (SI Appendix, Table S3). All cases were subjected to IHC for L1CAM, a well-established marker for EMT in human ECs (58, 59). Notably, only Fbxw7-mutant tumors expressed L1CAM, and all of these were also p53-positive by IHC, although not all p53-positive cases harbored Fbxw7 mutations (SI Appendix, Fig. S8 C and D). These results support the notion that Fbxw7-mutant adenocarcinomas have distinct properties and exhibit an EMT signature that distinguishes them from Fbxw7-wt adenocarcinomas.

Discussion

These studies implicate Fbxw7 as a particularly important driver of UCs. While Fbxw7 has been previously documented to undergo recurrent mutations in UCs, and could be surmised to be a tumor suppressor in UCs, prior studies had not pointed to Fbxw7 as a specific driver of the unique clinicopathologic phenotypes associated with UCS (6, 21). Here, by taking advantage of defined genetic model systems, we showed that tumorigenesis driven by concurrent Fbxw7 and Pten mutations progresses in a stereotypical manner. Neoplasia begins as well-defined EINs, which progress to well-differentiated endometrioid adenocarcinomas and then evolve into UCS with distinct epithelial and mesenchymal elements. This progression recapitulates many aspects of human endometrial carcinogenesis, including frequent squamous differentiation, heterologous differentiation, and aggressive clinical behavior with widespread metastasis upon progression to UCS.

This model provided a unique opportunity to identify cooperating oncogenic events. Specifically, although EINs were widespread, invasive carcinomas tended to arise focally (albeit with widespread metastasis upon progression to UCS. However, to begin to explore this question, we studied a defined set of uterine adenocarcinomas subjected to next-generation sequencing of a comprehensive cancer gene panel (UNCseq) (57). We studied 28 cases harboring canonical Fbxw7 mutations (WD40 hotspot or definite null mutations) and 20 cases without Fbxw7 mutations (SI Appendix, Table S3). All cases were subjected to IHC for L1CAM, a well-established marker for EMT in human ECs (58, 59). Notably, only Fbxw7-mutant tumors expressed L1CAM, and all of these were also p53-positive by IHC, although not all p53-positive cases harbored Fbxw7 mutations (SI Appendix, Fig. S8 C and D). These results support the notion that Fbxw7-mutant adenocarcinomas have distinct properties and exhibit an EMT signature that distinguishes them from Fbxw7-wt adenocarcinomas.

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or preinvasive. There are no strict histologic criteria for UCS (2), but a single sarcoma focus >1 mm² is considered sufficient even in very large tumors (69), implying that progression from a preexisting adenocarcinoma is a regular occurrence. Fhox7 mutations are more frequent in UCS, but also occur in usual-type endometrioid adenocarcinomas, raising questions as to the natural progression of such tumors. Most uterine cancers are diagnosed relatively early due to vaginal bleeding and are effectively cured by hysterectomy. It is intriguing to speculate that Fhox7-mutant endometrioid adenocarcinomas (were they left in the body) might have a propensity to progress into UCS, perhaps through metastasis into endometriotic adenocarcinomas, raising questions as to the natural history, precursors, and histologic intermediates for UCS.

Materials and Methods

A detailed description of mouse stocks and of allele and plasmid generation, antibodies used, cell line isolation, culture methods, tissue processing and staining, nucleic acid methods, Western blotting, and live cell imaging methods are provided in SI Appendix, Materials and Methods.

Animal Experimentation. All experiments were performed under a protocol approved by the University of Texas Southwestern (UTSW) Medical Center Institutional Animal Care and Use Committee.

Statistical Analysis. Error bars represent ±SEM; P values were calculated with GraphPad Prism V7.04 using the statistical tests described for each experiment unless otherwise indicated. For survival analysis, animals of the specified genotypes were assigned into aging cohorts at the beginning of the experiment and allowed to age undisturbed. Scoring of immunohistochemical staining intensity was performed by an investigator blinded as to the underlying genotype(s).

Data Availability. All data in this manuscript are freely available. The Sequence Read Archive (NCBI) accession number for the RNA-seq data is SRR5613208. The GEO (NCBI) accession number for the DNA sequencing data is GSE138490.

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