Glandular defects in the mouse uterus with sustained activation of TGF-beta signaling is associated with altered differentiation of endometrial stromal cells and formation of stromal compartment

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Abstract

Uterine gland development, also known as adenogenesis, is a key uterine morphogenic process indispensable for normal uterine function and fertility. Our earlier studies have reported that overactivation of TGFβ receptor 1 (TGFBR1) in the mouse uterus using progesterone receptor (Pgr)-Cre recombinase causes female infertility, defective decidualization, and reduced uterine gland formation, a developmental milestone of postnatal uterus. To understand mechanisms that underpin the disrupted uterine gland formation in mice with sustained activation of TGFBR1, we raised the question of whether early postnatal adenogenesis was compromised in these mice. Experiments were designed using mice with constitutive activation of TGFBR1 driven by Pgr-Cre to determine the timing of adenogenic defects and potential mechanisms associated with dysregulation of adenogenesis-associated genes. Notably, the adenogenic defects occurred in spite of the increased proliferation of uterine luminal epithelial cells, accompanied by increased expression of genes associated with fibrotic changes. Moreover, the adenogenic defects were alleviated in mice where TGFBR1 was activated in presumably half of the complement of uterine cells. Our results...
suggest that altered differentiation of endometrial stromal cells and formation of stromal compartment promote adenogenic defects.

Introduction

An increasing number of reproductive-aged women face pregnancy loss and infertility, some of which are associated with uterine dysfunction. Transforming growth factor beta (TGFβ) superfamily members are evolutionarily conserved and fundamental regulators of cell growth and differentiation. Critical roles of TGFβ superfamily members in female reproduction including post-implantation uterine function and pregnancy maintenance have been demonstrated, with the application of genetically engineered mouse models [1–3].

TGFβ ligands (TGFβ1-3) signal through a receptor complex consisting of TGFβ type 1 and type 2 receptors (TGFBR1/TGFBR2). Canonically, activated receptors impinge on receptor-regulated SMADs (R-SMADs) and SMAD4, the common SMAD, to elicit biological responses in target cells through the regulation of gene transcription [4]. The in vivo function of TGFβ signaling in the uterus remains incompletely understood [5–9]. Recent advances in tissue/cell specific targeting technology using Cre-LoxP system have been effective in deciphering gene function in reproduction and development [1, 3, 10, 11].

By taking advantage of TGFBR1 conditional loss-of-function and gain-of-function mouse models, we have gained a new understanding of TGFβ signaling in female reproductive tract development and function [3, 12, 13]. Because balanced TGFβ signaling controls homeostatic cellular processes, our approach to use both loss-of-function and gain-of-function mouse models is complementary and beneficial to define the role of TGFβ signaling in both physiologic and pathologic conditions. In an earlier report, we generated a mouse model harboring a constitutively active TGFBR1 allele, the expression of which was conditionally driven by the progesterone receptor (Pgr)-Cre recombinase [13]. These mice were sterile and developed several phenotypic abnormalities including enlarged myometrial compartment, disorganized myometrium, reduced stromal compartment and impaired uterine gland formation [13].

Uterine gland development, also known as adenogenesis, is a key uterine morphogenic process indispensable for normal uterine function [14–18]. Recent studies from the Spencer laboratory showed that uterine glands play critical roles in embryo implantation and endometrial decidualization, essential events for a successful pregnancy [19]. The mechanisms underlying the development of uterine glands are not well delineated. Adenogenesis is a complex physiological event whereby a number of genes, including, but not limited to forkhead box A2 (Foxa2), wingless-type MMTV integration site family member 4 (Wnt4), Wnt5a, Wnt7a and E-cadherin (Cdh1), are known to be essential [18, 20–27]. Conditional deletion of FOXA2, a uterine gland-specific transcription factor, causes a marked reduction in the number of uterine glands [18, 20]. WNT signaling plays critical roles in adenogenesis. For example, deletion of Wnt7a or Wnt4 in the mouse uterus results in lack of uterine glands or reduced number of uterine glands [21, 25]. Conditional ablation of CDH1, a cell-cell adhesion molecule, leads to loss of uterine glands in the neonatal uterus [26]. Hormone-related activities are also known to affect adenogenesis. Chronic exposure to progesterin in ewes from birth blocks adenogenesis, leading to ablation of endometrial glands [28]. Progesterone treatment also suppresses uterine gland development in the neonatal mouse uterus, along with reduced proliferation of the luminal epithelial cells [29]. In addition, epithelial-mesenchymal interactions are important for female reproductive tract development [30], and its involvement in adenogenesis requires further investigation.
The role of TGFβ signaling in the adenogenic process is poorly defined. Of note, adenogenic defects have not been reported in Tgfbr1 conditionally ablated mice using Pgr-Cre [31, 32], indicating that TGFBR1 is not essential for adenogenesis. However, constitutively active TGFBR1 in the mouse uterus severely impaired uterine gland formation [13]. To understand potential mechanisms that underpin the disrupted uterine gland formation in mice with sustained activation of TGFBR1, we determined whether postnatal adenogenic process was compromised at histological, cellular, and molecular perspectives. Additionally, we generated a complementary mouse model, in which activation of TGFBR1 occurs presumably in half of the complement of uterine cells to further understand the effect of TGFBR1 activation on adenogenesis. Our results show that overactivation of TGFBR1 impairs the differentiation of endometrial stromal cells and the formation of an integral stromal compartment, resulting in adenogenic defects. This finding underscores the importance of stromal-epithelial interaction during uterine development.

Materials and methods

Ethics statement

Mice were housed in the Texas A&M University Laboratory Animal Resources and Research (LARR) facility from the Comparative Medicine Program under a 12-hour light, 12-hour dark cycle and had access to the food and water ad libitum. Mice were cared by experienced veterinary technicians and trained research staff. Animal use protocol for this study was approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University (protocol numbers: 2014–0346 & 2016–0198). Animal manipulation and handling were performed according to the Guide for the Care and Use of Laboratory Animals guideline of National Institute of Health and Texas A&M IACUC.

Animals

TGFBR1\textsuperscript{CA} flox allele was constructed by targeting the constitutively active TGFBR1 into hypoxanthine phosphoribosyl-transferase (Hprt) locus, and the resultant mice harbor a functional Hprt gene [33]. Generation of Pgr-Cre mice and TGFBR1\textsuperscript{CA flox/flox; Pgr\textsuperscript{Cre/+}} mice was detailed elsewhere [13, 32]. To generate TGFBR1\textsuperscript{CA flox/++; Pgr\textsuperscript{Cre/+}} mice, the TGFBR1\textsuperscript{CA flox/flox} mice were bred with Pgr\textsuperscript{Cre/+} mice. Mice were genotyped by genomic polymerase chain reaction (PCR) to determine the presence of TGFBR1\textsuperscript{CA} flox allele and Pgr-Cre using specific primers reported previously [1, 33].

Sample preparation

Uterine samples were collected from control and experimental groups at different timepoints including D5, D7, D15, D21 and D31 and fixed in 10% neutral buffered formalin for immunohistochemistry and immunofluorescence analysis or homogenized in lysis buffer supplemented by RNeasy Mini Kit (Qiagen) and stored at −80°C until use.

Immunofluorescence and immunohistochemistry

Tissue processing and embedding were carried out using the histology core facility of the Department of Veterinary Integrative Biosciences at Texas A&M University. Paraffin sections (5 μm) were used for both immunofluorescence and immunohistochemistry [3, 12]. Briefly, sections were deparaffinized in xylene and rehydrated in graded ethanol. Then the slides were subject to antigen retrieval using 10 mM citrate buffer (pH 6.0). For immunofluorescence microscopy, sections were blocked with 5% bovine serum albumin (BSA) and incubated at
4˚C overnight with antibodies including mouse anti-alpha smooth muscle actin (ACTA2; ab76549; 1:1600; Abcam), rat anti-cytokeratin 8 (KRT8) (1:100; TROMA-I; Developmental Studies Hybridoma Bank), rabbit anti- FOXA2 (1:250; ab108422; Abcam), goat anti-forkhead box L2 (FOXL2; ab5096; 1:1500; Abcam) and rabbit anti-antigen identified by monoclonal antibody Ki 67 (Ki67; ab16667; 1:200; Abcam). Subsequent incubation of the sections with secondary antibodies conjugated with Alexa Fluor 488 or 594 (1:400; Invitrogen) was performed at room temperature (RT) for 1 h. The slides were mounted using ProLong Gold Slow fade media with DAPI (Invitrogen). Immunofluorescence signals were examined under an IX73 microscope interfaced with an XM10 CCD camera and cellSens Digital Imaging Software (Olympus). For immunohistochemical analysis, sections following antigen retrieval were treated with 3% H2O2 to quench endogenous peroxidase activity. Then, the sections were blocked with 5% non-immune serum and sequentially incubated with primary antibodies including rat anti-KRT8 (1:200; TROMA-I; Developmental Studies Hybridoma Bank), rabbit anti-estrogen receptor α (ERα; sc-542: 1:1000; Santa Cruz), rabbit anti-PGR (1:50; MA5-14505; Thermo Scientific), rabbit anti-calponin (CNN1; 1:500; #04589; EMD Millipore), rabbit anti-vimentin (VIM; 1:200; #5741; Cell Signaling Technology) and rabbit anti-collagen I (COL-I; 1:200; ab34710; Abcam), secondary antibody and Avidin/Biotin Complex (ABC; Vector Laboratories). The signals were developed using NovaRED Peroxidase Substrate Kit (Vector Laboratories). The sections were counterstained with hematoxylin and mounted with Permount (Fisher Scientific). Isotype-matched IgGs were included as negative controls.

**Trichrome staining**

Trichrome Staining was performed using Trichrome Stain Kit from Abcam (ab150686) based on manufacturer’s instructions. In brief, serial paraffin sections were deparaffinized and rehydrated. Post fixation was conducted using Bouin’s Fluid for 60 min. Slides were then rinsed and incubated with equal volumes of Weigert’s A and Weigert’s B Iron hematoxylin for 5 min. After being washed, slides were sequentially incubated with Biebrich Scarlet/Acid Fuchsin Solution and Phosphomolybdic/Phosphotungstic Acid for 3 and 15 min, respectively. Slides were stained with Aniline Blue Solution for 10 min, followed by 3 min incubation with Acetic Acid Solution (1%). Then, slides were dehydrated and mounted.

**Human endometrial stromal cell culture and treatment**

Human endometrial stromal cells (T-HESC; ATCC No. CRL-4003) [34] were cultured in phenol red-free DMEM/F12 supplemented with 10% charcoal-stripped fetal bovine serum (FBS; HyClone), 1% ITS + Premix (BD), 500 ng/ml puromycin (Invitrogen) and 100 U/ml Penicillin and 100 μg/ml Streptomycin. Cells were maintained at 37˚C supplemented with 5% CO2. Authentication of cells was performed by ATCC using short tandem repeat (STR) analysis. The cells were further verified using a functional assay to demonstrate their ability to undergo decidualization response via increasing the expression of insulin like growth factor binding protein 1 (IGFBP1) mRNA upon treatment with 8-bromoadenosine 3’,5’-cyclic monophosphate (8-Br-cAMP) (0.5 mM) (S1 Fig). For TGFB1 treatment, the cells were serum-starved overnight and then treated with vehicle (VEHL) or TGFB1 (0.1–10 ng/ml; R&D) for 24 h. Cells were collected and total RNA and proteins isolated as described below.

**RNA isolation, reverse-transcription, and real-time PCR**

Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instruction, with on-column DNase digestion performed to eliminate potential DNA
contamination. RNA was dissolved in ribonuclease-free water and quantified using a NanoDrop Spectrophotometer ND 1000 (NanoDrop Technologies).

Reverse transcription (RT) and real-time RT-PCR analysis using CFX Connect real-time PCR Detection System (Bio-Rad) and SYBR green were conducted as described [12]. The assays were performed in duplicate for each sample. Primers were synthesized based on sequences from published reports [17, 26, 35–39], PrimerBank and RTPRimerDB [40, 41] (Table 1). TaqMan gene expression assays were conducted using TaqMan probes for Foxa2 (Mm01976556_s1), WAP four-disulfide core domain 3 (Wfdec3; Mm01243777_m1), chemokine (C-X-C motif) ligand 15 (Cxcl15; Mm00441263_m1), ribosomal protein L19 (Rpl19; Mm02601633_g1), IGFBP1 (Hs00236877_m1), integrin subunit alpha 1 (ITGA1; Hs00235006_m1), collagen type I alpha 1 chain (COL1A1; Hs00164004_m1) and RPL19 (Hs02338565_gH) based on manufacturer’s instruction (Thermo Scientific). The average cycle threshold (CT) values were calculated and DDCT method was used to determine relative gene expression levels [42].

### Western blotting

Protein samples were prepared from T-HESCs using radioimmunoprecipitation assay (RIPA) buffer. Cell lysates were kept on ice for 30 mins, followed by sonication and centrifugation. Supernatants containing soluble proteins were quantified using bicinchoninic acid method (Thermo Fisher Scientific). Approximately 15 μg of proteins were loaded onto 12% MiniPROTEAN TGX Precast Gels (Bio-Rad). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes by using Bio-Rad Trans-Blot Turbo Transfer System. The PVDF membranes were then blocked with 5% non-fat milk and incubated with rabbit anti-COL-1 (1:1000; ab34710; Abcam), rabbit anti- connective tissue growth factor (CTGF; 1:1000; ab6992; Abcam), rabbit anti-ACTA2 (1:4000; #19245; Cell Signaling Technology), mouse anti-ITGA1 (1:250; sc-271034; Santa Cruz) and rabbit anti-GAPDH (1:1000; # 2118; Cell Signaling Technology) overnight at 4˚C. Then, membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated antibody (1:20,000) for 1 h at room temperature. Following antibody incubation and extensive washes, Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) was added to the membranes for signal detection. Images were developed using a Bio-Rad ChemiDoc imaging system.

### Statistical analysis

Comparisons of two means were made using student t-test. One way analysis of variance (ANOVA) was used to determine significance among multiple groups followed by Tukey’s HSD. Data are shown as mean ± SEM. Significance was reported at *P < 0.05, **P < 0.01 and ***P < 0.001 or indicated using different letters (P < 0.05).

### Results

**Sustained activation of TGFB signaling in the uterus leads to defective adenogenesis at early postnatal development**

Our earlier study using mice harboring constitutively active TGFBR1 in the uterus (TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup>) has demonstrated several phenotypic changes including disorganized myometrium, enlarged myometrial but reduced stromal compartment and reduced uterine gland formation [13]. This report is to further understand mechanisms contributing to defective uterine gland formation. Since the mouse uterus acquires basic structure by postnatal day 15 (D15), we performed immunostaining of KRT8, an epithelial cell marker,
### Table 1. Sequences of real-time RT-PCR primers.

| Name   | Sequence (5'-3') | Reverse | Reference          |
|--------|------------------|---------|--------------------|
| Col1a1 | Forward          | GCTCCTCTTAAAGGGCCACATT | PrimerBank ID 34328108a |
|        | Reverse          | CCAAGGTCTTCACATGTTGGG |                        |
| Itga1  | Forward          | CTTCCCTTGGAAGTGAGTCA  | PrimerBank ID 26343427a |
|        | Reverse          | AGATTTGCCCTGGATGTTAGA |                        |
| Itgb1  | Forward          | ATGCCCAACCTTGCGGAGAAT | PrimerBank ID 52722a   |
|        | Reverse          | GCCATCGGATTGCGTGTGAT   |                        |
| Lama1  | Forward          | CCGGTCTCTGAGCCACGTCA  | [35]                   |
|        | Reverse          | GCCATCGGATTGCGTGTGAT   |                        |
| Acta2  | Forward          | GTCCACGACATCGAGAATTA  | PrimerBank ID 6671507a |
|        | Reverse          | TCGAGATACCTACGCGTACAG |                        |
| Wnt4   | Forward          | CATCGAGGGATGCACATACCA | [26]                   |
|        | Reverse          | GGCGAGCTGTCTACCTGTGG  |                        |
| Wnt5a  | Forward          | GGCGAAAGGGTGGACATAG   | [26]                   |
|        | Reverse          | GGCGAAAGGGTGGACATAG   |                        |
| Wnt7a  | Forward          | GCAGTGTGCTGCGACAGAG   | RTPrimerDB ID 3399     |
|        | Reverse          | TTTGCTGCGATTGGTGACATT |                        |
| Wnt11  | Forward          | GCCATGAGGCCTGGTGATAG  | [26]                   |
|        | Reverse          | GATGGGTGACTGATGTTG    |                        |
| Wnt16  | Forward          | CAGGGCAACTGAGATGTTG   | PrimerBank ID 2635574a |
|        | Reverse          | CTAGGCACGAGTGACAGTT   |                        |
| Fzd6   | Forward          | GGCAGTTGGTCTCTGTGTT   | [26]                   |
|        | Reverse          | CCAGAGGCAAGAGGGACAGAT |                        |
| Fzd10  | Forward          | TGCTCTGAGCGGCTGGT    | [17]                   |
|        | Reverse          | GATGAAGGAAAGTGCCGAGTG |                        |
| Ctnnb1 | Forward          | ATGGAGCGCCAGGACAGAAGC | PrimerBank ID 6671684a |
|        | Reverse          | CTGGCCACTCAGGGAGGAG   |                        |
| Sfrp1  | Forward          | CAAGGTGCGTACAGAAGAT  | PrimerBank ID 7305481a |
|        | Reverse          | GGCAGTAGAACGCGAAGAC   |                        |
| Sfrp2  | Forward          | CGTGGGTTCTTCTCTCTTG   | [38]; Primerbank ID 667795a |
|        | Reverse          | GTTTCTGCTGATGAGCCG    |                        |
| Sfrp3  | Forward          | ATTTGGTGTTTCTGATCCTGT | [39]                   |
|        | Reverse          | CGTTCTCTCAAAATGCTTCC |                        |
| Sfrp4  | Forward          | AGAAGCTCATACAGTTGGAA  | PrimerBank ID 7710094a |
|        | Reverse          | GTTACCTGCGATCGTGCGGA  |                        |
| Sfrp5  | Forward          | CACTGCACAGTTCCCCCA    | PrimerBank ID 3156042a |
|        | Reverse          | TCTTCTCCATGAGGGCAGAG  |                        |
| Foxd2  | Forward          | GCTACCAGCAGCCCCAGACAC | [36]                   |
|        | Reverse          | GTGTGCTCCCGCTCCCTCTG  |                        |
| Vim    | Forward          | GCTGGCAGAGGAGGTTTGGGA | PrimerBank ID 227430362c3 |
|        | Reverse          | CCACCTTCTGGTCAAGGTAAG |                        |
| Cd10   | Forward          | CTCTCTGTGCTCTCTGTGCTC | PrimerBank ID 3154325a |
|        | Reverse          | CAGAGGGGCTGTGCATGGT    |                        |
| ACTA2  | Forward          | TCAGTCCAGCCAGATTTGAT  | [37]                   |
|        | Reverse          | CAGCAGATGGCGCATTGTT    |                        |
| CTGF   | Forward          | TTGGCCAGACCCAACTATG   | RTPrimerDB ID 596      |
|        | Reverse          | CAGGAGGCGTGTGTCATGGT   |                        |

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at this timepoint to test the hypothesis that reduction of uterine glands in TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) mice was caused by defective adenogenesis. Our results showed a marked reduction of uterine glands in mice with constitutively active TGFBR1 at D15 compared with age-matched controls (Fig 1A–1D). Consistent with the impaired uterine gland formation, expression of mRNA transcripts for uterine gland specific genes including Foxa2, Wfdc3 and Cxcl15 was significantly decreased in TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) uteri compared with controls (Fig 1E).

To determine how constitutively active TGFBR1 affected early adenogenic event, we performed immunofluorescence microscopy using antibodies directed to KRT8 and ACTA2 at D5, D7 and D15. As expected, uterine glands were not visible at D5 in either control or TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) mice (Fig 2A and 2C). Epithelial invagination occurred at D7 in the controls (Fig 2E), whereas this histological change was less evident in the TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) uterus (Fig 2G). By D15, abundant uterine glands were detected in the control uterus (Fig 2I). In contrast, uterine glands were sparse in TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) mice (Fig 2K), confirming the aforementioned immunohistochemistry finding. FOXA2, a marker specific for glandular epithelia [43] and key regulator of uterine gland development and function [18, 27], was used to verify the identity of uterine glands. Immunofluorescence analysis of FOXA2 revealed the presence of abundant uterine glands in the control but not TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) mice (Fig 2M–2P). Further analysis of uterine glands per cross section confirmed that the number of uterine glands in the TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) mice was reduced compared with that of controls (0.5 ± 0.0 versus 10.2 ± 3.0; n = 3; P < 0.05).

Uterine smooth muscle cells differentiate and form myometrial layers after birth in mice. ACTA2 was used to identify myometrial compartment. In the control uterus, well organized myometrial layers and endometrial compartment were visualized by ACTA2 staining from D5 to D15 (Fig 2B, 2F and 2J). However, distinct endometrial and myometrial compartments could not be identified in TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) uteri at D5 and D7 using ACTA2 staining (Fig 2D and 2H). Instead, ACTA2-positive cells were in close proximity with luminal epithelial cells in the TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) uterus (Fig 2D and 2H), which was in sharp contrast to the control uteri (Fig 2B and 2F). At D15, an endometrial stromal compartment was visible in the TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) uterus, surrounded by disoriented ACTA2-positive cells. Disorganized uterine smooth muscle formation in the uteri of TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) mice at D5 and D15 was further verified using immunostaining of CNN1 (Fig 3A–3D) and abnormal development of endometrial compartment was evidenced by immunostaining of anti-VIM (Fig 3E–3H). Specific immunoreactive signals were not observed in negative controls using isotype-matched IgG (Fig 3I–3L). To further determine whether activation of TGFβ signaling modulated ER and PGR to shape a distinct developmental trajectory, we examined the expression of ER and PGR in the uteri of both control and TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) mice at D5 and D15 using immunohistochemistry. Our results showed a similar expression pattern of ER and PGR between control and TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) mice (S2 Fig). Collectively, enhanced TGFβ signaling in the mouse uterus negatively impacts adenogenesis during early postnatal uterine development, coinciding with altered differentiation of endometrial stromal cells and formation of endometrial compartment.

**Molecular analysis of genes associated with adenogenesis and uterine development in TGFBR1 constitutively active uterus**

To determine the potential mechanism underlying adenogenic defects observed in TGFBR1\(^{CA}\) flox/flox; Pgr\(^{Cre/+}\) uterus, we compared the transcript levels of genes known to be
involved in adenogenesis and uterine development using uterine samples from control and 
TGFBR1\( ^{CA} \) flox/flox; Pgr\(^{Cre/+}\) mice at D7, D15 and D31. Candidate genes included 
Wnt4, Wnt5a, Wnt7a, Wnt11, Wnt16, catenin beta 1 (Ctnnb1), frizzled homolog 6 (Fzd6), and 
Fzd10 [44, 45]. Significant changes in the expression of the above genes were not found in 
TGFBR1\( ^{CA} \) flox/flox; Pgr\(^{Cre/+}\) uteri at D7 except Wnt11 (Fig 4D). At D15, mRNA levels of 
Wnt4 (Fig 4A), Wnt7a (Fig 4C) and Ctnnb1 (Fig 4F) were increased, whereas expression of 
Wnt11 (Fig 4D) was reduced, in TGFBR1\( ^{CA} \) flox/flox; Pgr\(^{Cre/+}\) uteri compared with age-
matched controls. At D31, a significant difference was only detected in Wnt4 (Fig 4A) and 
Wnt16 (Fig 4E) mRNA expression between control and TGFBR1\( ^{CA} \) flox/flox; Pgr\(^{Cre/+}\) uteri.

As secreted Frizzled related proteins (SFRPs) can bind to WNT or Frizzled membrane
receptors to negatively modulate WNT signaling activity [46], we determined the mRNA expression of Sfrp1-5 in control and TGFBR1\textsuperscript{CA flox/flox; Pgr\textsuperscript{Cre/+}} uteri at D7, D15 and D31. Results are shown in Fig 4I–4M. Differential changes of the Sfrp genes in the uteri were observed between control and TGFBR1\textsuperscript{CA flox/flox; Pgr\textsuperscript{Cre/+}} mice among different developmental stages. The results revealed complex alterations of Sfrp expression in the uteri of TGFBR1\textsuperscript{CA flox/flox; Pgr\textsuperscript{Cre/+}} mice versus controls. These findings suggest a potential link between the dysregulation of adenogenic gene expression and the defective adenogenesis in TGFBR1\textsuperscript{CA flox/flox; Pgr\textsuperscript{Cre/+}} mice.

Foxl2, a critical gene in ovarian development and function [47], has been reported to be expressed in the uterus and required for uterine maturation [48]. Because conditional deletion of Foxl2 using Pgr-Cre also shows a similarly enlarged ACTA2-positive component [13, 48], we sought to determine whether the defective cellular differentiation in the TGFBR1\textsuperscript{CA flox/flox;
Pgr<sup>Cre/+</sup> uterus was linked to altered FOXL2 expression. Our real-time PCR assay showed comparable uterine mRNA levels of Foxl2 between control and TGFBR1<sup>CA flox/flox</sup>; Pgr<sup>Cre/+</sup> mice at both D7 and D15, despite the reduction (or trended reduction) of expression of stromal cell markers [49], cluster of differentiation 10 (Cd10) and vimentin (Vim) at D15 (Fig 5A). Immunofluorescence showed that FOXL2 was mainly expressed in uterine stromal cells in control mice (Fig 5B). In the TGFBR1<sup>CA flox/flox</sup>; Pgr<sup>Cre/+</sup> mice, comparable FOXL2 signals were detected in the stromal compartment (Fig 5C). Negative controls where FOXL2 antibody was replaced by isotype-matched IgG are depicted in Fig 5D and 5E. Positive controls using wild type ovaries showed specific staining of FOXL2 in ovarian granulosa cells (Fig 5F and 5G). This result indicates that overactivation of TGFBR1 may function independently of FOXL2 or TGFBR1 functions downstream of FOXL2 in the mouse uterus.

### Luminal epithelial cell proliferation is increased in TGFBR1 constitutively active uterus

Epithelial proliferation may play a permissive role in uterine adenogenesis [29, 50]. Thus, impaired luminal epithelial cell proliferation could potentially lead to the observed adenogenic defects. To determine whether luminal epithelial cell proliferation was altered in TGFBR1<sup>CA flox/flox</sup>; Pgr<sup>Cre/+</sup> uteri, we examined the proliferative status of uterine luminal...
epithelial cells at D7 and D21 in control and TGFBR1\textsuperscript{CA flox/flox}, Pgr\textsuperscript{Cre/+} mice using immunostaining of Ki67, a cell proliferation marker. Our results showed that uterine luminal epithelial cells were highly proliferative at D7 in both control and TGFBR1\textsuperscript{CA flox/flox}, Pgr\textsuperscript{Cre/+} uteri (Fig 6A–6D). The proliferation of uterine epithelial cells in D21 control mice was low (Fig 6E, 6F and 6I), consistent with a previous report \cite{29}. However, abundant Ki67-positive cells were present in the luminal epithelia of TGFBR1\textsuperscript{CA flox/flox}, Pgr\textsuperscript{Cre/+} uteri (Fig 6G, 6H and 6J). Further quantitative analysis did not reveal a difference in the number of Ki67-positive luminal epithelial cells between control and TGFBR1\textsuperscript{CA flox/flox}, Pgr\textsuperscript{Cre/+} uteri at D7, but showed that the number of Ki67-positive luminal epithelial cells was increased in TGFBR1\textsuperscript{CA flox/flox}, Pgr\textsuperscript{Cre/+} uteri versus controls at D21 (Fig 6K). Therefore, sustained
activation of TGFBR1 in the mouse uterus does not impede uterine luminal epithelial cell proliferation.

**Fibrotic changes in the TGFBR1 constitutively active uterus**

Consistent with the well-established role of TGFβ ligands as fibrotic proteins [51], transcript levels for genes encoding collagen and laminin (Col1a1 and Lama1) were significantly increased in TGFBR1CA flox/flox; Pgr Cre/+ uteri compared with controls at D7 and/or D31 (Fig 7A–7C). Integrins link extracellular matrix (ECM) to cytoskeleton. Here we demonstrated that the mRNA expression of Itga1 and Itgb1 was increased in the uteri with overactivation of TGFBR1 (Fig 7A and 7B). To visualize collagen-containing fibers in the uterus, we performed Trichrome staining using uteri from both control and TGFBR1CA flox/flox; Pgr Cre/+ mice at 1 month of age. Results showed increased blue collagen fibers in the stromal compartment of TGFBR1CA flox/flox; Pgr Cre/+ mice compared with controls (Fig 7D and 7E). Immunohistochemistry using anti-COL-1 antibody also revealed increased collagen protein expression in TGFBR1CA flox/flox; Pgr Cre/+ uteri versus controls (Fig 7F and 7G). Negative controls using
rabbit IgG are shown in Fig 7H and 7I. These data suggest that enhanced TGFB signaling leads to fibrotic changes in mouse endometrium. To independently test the role of TGFB signaling in the regulation of the fibrotic genes, we used human uterine stromal cells and showed that TGFB1 (0.1–10 ng/ml) treatment significantly increased the mRNA levels of ACTA2, CTGF [52], ITGA1 and COL1A1 after 24 h of treatment (Fig 8A–8D). Western blot analysis confirmed the stimulatory effect of TGFB1 on the protein expression of ACTA2, CTGF, ITGA1 and COL-1 (Fig 8E and S3 Fig). These results suggest that chronic activation of TGFBR1 leads to fibrotic changes in endometrial stromal compartment, which impairs endometrial cell function.

Mosaic activation of TGFBR1 in the mouse uterus alleviates adenogenic defects

The aforementioned uterine fibrotic changes and the unimpeded proliferation of epithelial cells suggest that the adenogenic defects observed in TGFBR1CA flox/flox; PgrCre/− mice are caused by altered uterine stromal cell differentiation and formation of endometrial stromal cell compartment. We anticipated that a reduction of the number of endometrial stromal cells expressing the TGFBR1CA transgene would alleviate the adenogenic defects. Therefore, we generated a mouse model with activation of TGFBR1 in presumably half of the complement of uterine cells (i.e., TGFBR1CA flox/++; PgrCre/−) due to the targeting of TGFBR1CA to the X-linked
Hprt locus and X-chromosome inactivation in the females during development [33, 53].

Expression of TGFBR1 mRNA levels in the uteri from TGFBR1CA flox/+; PgrCre/+ mice at the age of 1 month were analyzed using real-time PCR, demonstrating the expression of the transgene (S4 Fig). As expected, adenogenesis occurred in TGFBR1CA flox/+; PgrCre/+ uteri. n = 4 per group. Data are means ± SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001. (D and E) Trichrome staining of uteri from control and TGFBR1CA flox/+; PgrCre/+ mice at 1 month of age. Note increased blue staining in uterine stroma of TGFBR1CA flox/+; PgrCre/+ mice versus controls. (F-I) Immunohistochemistry of COL-1 in the uteri from control and TGFBR1CA flox/+; PgrCre/+ mice at 1 month of age. Negative controls using rabbit IgG are shown in (H) and (I). Three individual samples from each group were examined. Scale bar is representatively shown in (D) and equals 20 μm (D-I).

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Fig 7. Increased expression of extracellular matrix related genes and fibrotic changes in TGFBR1CA flox/flox; PgrCre/+ uteri. (A) Itga1 mRNA levels were elevated in TGFBR1CA flox/flox; PgrCre/+ uteri at D7. (B) Colla1, Itga1 and Itgb1 mRNA abundance was increased in TGFBR1CA flox/flox; PgrCre/+ uteri at D31. (C) Lama1 mRNA levels were increased in TGFBR1CA flox/flox; PgrCre/+ uteri. n = 4 per group. Data are means ± SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001. (D and E) Trichrome staining of uteri from control and TGFBR1CA flox/flox; PgrCre/+ mice at 1 month of age. Note increased blue staining in uterine stroma of TGFBR1CA flox/flox; PgrCre/+ mice versus controls. (F-I) Immunohistochemistry of COL-1 in the uteri from control and TGFBR1CA flox/flox; PgrCre/+ mice at 1 month of age. Negative controls using rabbit IgG are shown in (H) and (I). Three individual samples from each group were examined. Scale bar is representatively shown in (D) and equals 20 μm (D-I).

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Understanding defective uterine gland development in mice harboring constitutively active TGFBR1
Discussion

Toward the goal to defining the role of TGFB signaling in uterine function, we created a mouse model that harbors a constitutively active TGFBR1 in the uterus using Pgr-Cre recombinase in a previous report [13]. In addition to myometrial abnormality, uterine gland development was impaired in these mice. This study was to follow up the adenogenic defects in these mice and identify the underpinning cellular and molecular basis.

The uterus differentiates from Müllerian duct. In general, Müllerian duct differentiation is a highly coordinated event regulated by genes including, but not limited to, LIM homeobox protein 1 (LIM1), paired box 2 (Pax2), Wnt9b, Wnt5a and homeobox (Hox) genes [22, 54–57]. Adenogenesis, a physiologic process of uterine gland formation, occurs postnatailly in mice [50]. Recent studies from the Behringer laboratory demonstrated that uterine gland formation is a continuous process in mice [58]. The uterus contains simple epithelium and supporting mesenchyme, with no endometrial glands at birth. Adenogenesis occurs via invaginations of luminal epithelium by D6. Uterine glands are evident on D7, and the uterus acquires essential structures (i.e., myometrium and endometrium consisting of stroma and glands) by D15 [59, 60]. Therefore, we examined adenogenesis in TGFBR1CA flox/flox; PgrCre/+.
mice during the critical period of uterine gland formation and revealed abnormal uterine stromal cell differentiation and formation of uterine stromal compartment. The altered uterine cell differentiation suggests that uterine cells are sensitive to TGFBR1 overactivation. However, a potential contribution of altered uterine epithelial-mesenchymal interaction to the abnormal ACTA2 expression could not be excluded.

It has been reported that WNT pathway components including WNTs and CTNNB1 are crucial for uterine gland development [22, 23, 61, 62]. For examples, both WNT5A and WNT7A are required for uterine gland formation and WNT7A is also essential for normal patterning of the uterus during development [22, 23, 25]. WNT4, which is abundantly expressed in the stroma of neonatal mouse uterus [44], has also been implicated in uterine gland formation. A recent study suggests that DICER1 may be a regulator of uterine gland development, based on the observation that conditional knockout of Dicer1 using Pgr-Cre leads to loss of glandular epithelium in the uterus [63]. However, how loss of DICER1 contributes to this phenotype requires further investigation. TGFB signaling interacts with WNTs [64]. Therefore, we examined the expression of WNT pathway components in the TGFBR1 CA flox/flox; Pgr Cre/+ uterus. Interestingly, despite the altered cell properties, the expression of Ctnnb1 and a number of adenogenesis associated genes in the uteri of TGFBR1 CA flox/flox; Pgr Cre/+ mice was comparable to that of controls at D7, except a reduction of Wnt11 and Sfrp3 mRNA levels. Although Wnt11 is expressed in uterine epithelium, conditional ablation of Wnt11 does not impair uterine gland genesis [44], suggesting the adenogenic program may be functional at the beginning of adenogenesis. It was noteworthy that several Wnt and Sfrp
genes were dysregulated at D15 and/or D31. Although the specific role of WNT11 in uterine development is not clear, its reduction in the TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup> uteri could reflect the decreased epithelial components in these mice. It is intriguing, however, Wnt4, Wnt7a, Wnt16 and Ctnnb1 mRNA levels were increased in the TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup> uteri at D15. Since these genes are essential for normal uterine gland development [21–23, 25, 44, 61], it is tempting to speculate that their upregulation in the TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup> uteri may represent a compensatory mechanism.

Elegant work has shown that progesterone administration in a limited time window during postnatal uterine development results in depletion of endometrial glands, accompanied by inhibition of luminal epithelial cell proliferation [17, 29]. To determine whether the impaired adenogenesis in our model is potentially caused by compromised luminal epithelial cell proliferation, we assessed the proliferation status of uterine epithelial cells and demonstrated that the proliferation of luminal epithelial cells was not impeded at D7. In fact, increased number of Ki67-positive luminal epithelial cells was found in the TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup> uterus at D21. Thus, this finding further indicates that the adenogenic program may remain functional in the TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup> uterus at the beginning of uterine gland formation. However, dysregulation of Wnt pathway-related genes at D15 indicates that this program may be compromised with the progression of adenogenic process.

Uterine epithelial-mesenchymal interaction is critically important for uterine development and the specification and maintenance of the integrity of mesenchymal compartments [22, 65]. An interesting finding of this study was that uterine stromal cell differentiation was altered at an early developmental stage (i.e., D5) in the TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup> uteri, characterized by unrecognizable endometrial stromal compartment at D5 and D7. Consequently, ACTA2-positive cells were in close proximity with luminal epithelia in the TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup> mice, as is potentially detrimental to the formation of uterine glands. Although an endometrial stromal compartment was morphologically identifiable at D15, it was restricted and comprised sparse uterine glands. It has been well established that TGFB signaling is a potent driver of fibrotic responses in a variety of organs/tissues [51, 66, 67]. Our human endometrial cell culture experiment extends the findings from mice by revealing the regulation of several profibrogenic genes by TGFB signaling. We found that TGFB1 induces the expression of ACTA2, COL1A1, ITGA1 and CTGF, in line with previous reports that TGFB/SMAD signaling promotes fibrosis by inducing transcription of pro-fibrogenic genes [13, 68, 69]. Of note, CTGF is an essential mediator of tissue remodeling and fibrosis [52]. Upregulation of CTGF by TGFB in human endometrial cells is consistent with our previous report that Ctgf transcripts are increased in TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup> uteri [13]. It is thus plausible that the changes of the expression of genes encoding matrix proteins, integrins and smooth-muscle filament proteins in the TGFBR1<sup>CA</sup> flox/flox; Pgr<sup>Cre/+</sup> uteri may influence the matrix properties and promote the development of a smooth muscle-like barrier that is less permissive to uterine gland branching/formation compared to a normal uterine microenvironment where the “soil” for adenogenesis consists primarily of normal stromal cells. Furthermore, by taking advantage of a mouse model where TGFBR1<sup>CA</sup> transgene was expressed in presumably half of the complement of uterine cells, we found that adenogenesis occurred in these mice. Results from this model provided circumstantial evidence that impaired adenogenesis is caused by altered endometrial stroma property. This evidence supports the notion that abnormal mesenchymal cell differentiation caused by overactivation of TGFBR1 negatively impacts uterine microenvironment and adenogenesis. The model may serve as a tool to study the effect of activation of TGFBR1 in partial versus full complement of uterine cells. However, a potential caveat for this model is that reduction of cells expressing TGFBR1<sup>CA</sup> may occur not only in the stromal compartment but also in the epithelial lineage due to the known expression pattern of Pgr-Cre in
the uterus. This possibility needs to be further investigated. In addition, although the defects in early postnatal adenogenesis observed in mice harboring constitutively active TGFBR1 signify the importance of appropriately controlled TGFB signaling activity in uterine development, findings from this mouse model should not be interpreted as the physiologic role of TGFB signaling in adenogenesis.

In summary, our findings suggest that altered differentiation of uterine stromal cells and formation of endometrial stromal compartment resulting from sustained activation of TGFBR1 is a key contributing factor to the adenogenic defects in these mice. This study has potential implication in understanding the pathological role of TGFB signaling in uterine disease associated with endometrial dysfunction.

Supporting information

S1 Fig. Induction of IGFBP1 mRNA expression in THESC upon treatment with 8-bromoadenosine 3', 5'-cyclic monophosphate (8-Br-cAMP). THESCs were treated with vehicle (VEHL) or 8-Br-cAMP (0.5 mM) for 6 days. Four independent cell culture experiments were performed. Data are means ± SEM. **P < 0.01.

(TIF)

S2 Fig. Expression of ER and PGR in the uteri of mice with constitutively active TGFBR1 during early uterine development. (A-H) Immunohistochemical analysis of ER and PGR in the uteri of control and TGFBR1CA flox/flox; PgrCre/+ mice at D5 and D15. Three individual samples from each timepoint were examined. Scale bar is representatively shown in (A) and equals 50 μm (A-H).

(TIF)

S3 Fig. Uncropped western blot images. Full images for western blot shown in Fig 8E. First row shows the blots for ACTA2, CTGF, ITGA1 and COL-1 proteins, and the second row shows the corresponding GAPDH. Dashed boxes indicate target bands with expected molecular weights.

(TIF)

S4 Fig. Expression of TGFBR1CA in the uteri of TGFBR1CA flox/+; PgrCre/+ mice at the age of 1 month. n = 4 for control and n = 6 for TGFBR1CA flox/+; PgrCre/+ mice. Rpl19 was used as internal control. Data are means ± SEM. ***P < 0.001.

(TIF)

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