Tumor Susceptibility Gene 101 Is Required for the Maintenance of Uterine Epithelial Cells During Embryo Implantation

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Research Article

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Abstract

**Background:** The tumor susceptibility gene 101 (Tsg101), a component of the endosomal sorting complex required for transport (ESCRT) complex I, is involved in multiple biological processes involving endomembranous structures and the plasma membrane. The role of Tsg101 in the uterine epithelium was investigated in Tsg101 floxed mice crossed with Lactoferrin-iCre mice (Tsg101<sup>d/d</sup>).

**Methods:** Tsg101<sup>d/d</sup> mice were bred with stud male mice and the status of pregnancy was examined on days 4 and 6. Histological analyses were performed to examine the uterine architecture. Immunofluorescence staining of several markers was examined by confocal microscopy. Uterine epithelial cells (UECs) were isolated from Tsg101<sup>f/f</sup> and Tsg101<sup>d/d</sup> mice, and the expression of necroptosis effectors was examined by RT-PCR, western blotting, and immunofluorescence staining. UECs were also subjected to RNA expression profiling.

**Results:** Tsg101<sup>d/d</sup> female mice were subfertile with implantation failure, showing unattached blastocysts on day 6 of pregnancy. Histological and marker analyses revealed that some Tsg101<sup>d/d</sup> day 4 pregnant uteri showed a disintegrated uterine epithelial structure. Tsg101<sup>d/d</sup> UECs began to degenerate within 18 h of culture. In UECs, expression of necroptosis effectors, such as RIPK1, RIPK3, and MLKL were first confirmed. UECs responded to a stimulus to activate necroptosis and showed increased cell death.

**Conclusions:** Tsg101 deficiency in the uterine epithelium causes implantation failure, which accompanies epithelial defects. This study provides evidence that UECs harbor a necroptotic machinery that responds to death-inducing signals. Thus, Tsg101 expression in the uterine epithelium is required for normal pregnancy in mice.

**Background**

The endosomal sorting complex required for transport (ESCRT) complexes, ESCRT-0, -I, -II, and -III, act in sequence as key regulators of endosomal sorting and maturation. The ESCRT-I complex contains tumor susceptibility gene 101 (Tsg101), vacuolar protein sorting-associated protein 28 homolog Vsp28, Vsp37, and multivesicular body sorting factor 12 (Mvb12) proteins [1]. As a component of ESCRT-I, Tsg101 forms a complex with other ESCRT factors and is essential for the recruitment of subsequent ESCRT complexes [2]. ESCRT proteins are also required for the maintenance of epithelial cell polarity [3].

Tsg101 protein has a ubiquitin-interacting domain and downregulates ubiquitinated cell surface receptors and certain protein aggregates [4, 5]. It is also involved in cytokinesis and viral exit from infected cells [6, 7]. Systemic deletion of Tsg101 results in early embryonic death between embryonic days 5.5 and 6.5 due to defective cell proliferation [8]. Deletion of Tsg101 in mouse embryonic fibroblasts (MEFs) causes cell cycle arrest and cell death [9], suggesting a complex role for this gene [7].

Cell death is essential for various biological processes including basic pathophysiology and embryonic development [10]. Depending on the molecular pathways and morphological characteristics, cell death is
classified into several types, such as apoptosis, autophagy, and programmed necrosis (necroptosis) [11]. Necroptosis often begins with the activation of death receptors by cognate ligands, such as tumor necrosis factor α (TNFα), TNF related apoptosis-inducing ligand (TRAIL), and FAS ligand (FASL). Intracellular signaling follows involving receptor-interacting protein kinase (RIPK) 1, RIPK3, and mixed lineage kinase-like (MLKL) proteins [12]. Phosphorylated MLKL (pMLKL) proteins translocate to the plasma membrane and mediate membrane permeabilization [13]. MLKL activation results in Ca$^{2+}$ influx, which is rapidly followed by lipid scrambling of the plasma membrane. The damaged plasma membrane depends on certain ESCRT components to maintain integrity following MLKL activation. Charged multivesicular body protein 4B (CHMP4B) and other ESCRT factors produce small membrane vesicles to mend the plasma membrane during necroptosis [13]. Tsg101 promotes the translocation of ESCRT-III factors to the sites of membrane damage and counteracts plasma membrane rupture during necroptosis [13].

In mice, embryo implantation occurs around midnight on day 4 of pregnancy [14]. For this process to proceed successfully, the luminal epithelium undergoes steroid hormone-induced proliferation and differentiation, which renders it competent for embryo attachment [15]. The communication between an implantation-competent blastocyst and a receptive uterus is central to the implantation process and successful pregnancy, and any defect in this process results in implantation failure [15]. The uterine epithelium at the time of embryo implantation undergoes differentiation, expressing several factors involved in two-way interactions. The importance of epithelial polarity in embryo implantation has been demonstrated in a study examining the role of planar cell polarity signaling [16].

Lactoferrin (Ltf) encodes a non-heme iron-binding glycoprotein and is highly responsive to estrogen in the mouse uterus [17]. Ltf is expressed in the uterine epithelium of adult mice but not in immature mouse uteri [18]. A mouse Cre model taking advantage of this expression pattern is available as Ltf-iCre knock-in mice [19], in which iCre is expressed under the endogenous Ltf promoter. This Cre model efficiently recombines the floxed target gene, primarily in the uterine epithelium, in adult female mice and immature females after estrogen treatment [19]. In this study, we generated a uterine epithelium-specific Tsg101 deletion model by crossing Tsg101 floxed (Tsg101$^{f/f}$) mice with Ltf-iCre mice to examine its role in this cell type. Our results show that Tsg101 is required for the maintenance of the uterine epithelium and embryo implantation.

**Materials And Methods**

**Reagents**

17β-estradiol (E$_2$) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in sesame oil (Acros Organics). Equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) were purchased from Sigma-Aldrich.

**Mice**
All mice were maintained in accordance with the policies of the Konkuk University International Animal Care and Use Committee (IACUC). *Tsg101* floxed mice (*Tsg101*<sup>f/f</sup>) mice [8] were crossed with *Ltf-iCre* mice [19] to obtain *Ltf-iCre, Tsg101*<sup>f/f</sup> (*Tsg101*<sup>d/d</sup>) mice. *Tsg101*<sup>d/d</sup> mice were produced by crossing *Tsg101*<sup>f/f</sup> female mice with *Ltf-iCre, Tsg101*<sup>f/d</sup> male or *Ltf-iCre, Tsg101*<sup>f/f</sup> male mice. Genomic DNA was extracted from mouse tails using Gentra Puregene Mouse Tail kit (Qiagen, Hilden, Germany). Genotyping PCR for the floxed *Tsg101* and *Ltf-iCre* genes was performed using the primers shown in Table 1. This study was approved by the Konkuk University IACUC (approval number KU20036).
| Gene                  | Sequence (5′ – 3′)                                      | Annealing temperature (°C) | No. of cycles | Product size (bp) |
|-----------------------|--------------------------------------------------------|-----------------------------|---------------|------------------|
| **Tsg101 wildtype**   | F: CCG TGA TCT CTT GAT TCT TCT CC                      | 58                          | 35            | 482              |
|                       | R: CCT GCT CTT TAC TGA AGG CTC                         |                             |               |                  |
| **Tsg101 floxed**     | F: CCG TGA TCT CTT GAT TCT TCT CC                      | 58                          | 35            | 482              |
|                       | R: GAA ATC CAC CTG CCT CTG CTC                         |                             |               |                  |
| **Ltf<sup>Cre</sup>** transgene | F: AAC TAG CAC ACC TGG TTG AGG                         | 60.5                        | 10            | 215              |
|                       | R: CAG GTT TTG GTG CAC AGT CA                          |                             |               |                  |
| **Des**               | F: CAA AGG GGT TCT GAA GTC CA                          | 59                          | 28            | 198              |
|                       | R: GAA AAG TGG CTG GGT GTG AT                          |                             |               |                  |
| **Krt12**             | F: GTC TCA TCC CAG GTT CAG GA                          | 59                          | 26            | 231              |
|                       | R: TGC AAT GAA GAC CAG CAG AG                          |                             |               |                  |
| **Rpl7**              | F: TCA ATG GAG TAA GCC CAA AG                          | 59                          | 28            | 246              |
|                       | R: CAA GAG ACC GAG CAA TCA AG                          |                             |               |                  |
| **Tsg101**            | F: ATG GCG GTG TCC GAG AGT CAG                         | 55                          | 33            | 80               |
|                       | R: TTG ACA GTT TGA CGG ACG GT                          |                             |               |                  |
| **Ripk1**             | F: GAA GAC AGA CCT AGA CAG CGG                         | 58                          | 35            | 182              |
|                       | R: CCA GTA GCT TCA CCA CTC GAC                         |                             |               |                  |
| Gene | Sequence (5’ – 3’) | Annealing temperature (°C) | No. of cycles | Product size (bp) |
|------|-------------------|---------------------------|--------------|------------------|
| Ripk3 | F: CAC ATA CTT TAC CCT TCA GA  
R: TCA GAA CAG TTG TTG AAG AC | 58 | 35 | 172 |
| Mlk1 | F: GAC CAA ACT GAA GAC AAG TA  
R: CTC ACT ATT CCA ACA CTT TC | 57 | 35 | 114 |
| Aqp8 | F: GGG GCA GCC TTT GCC ATC GT  
R: AAG AGG CCA GCC AGG AGG GG | 59 | 28 | 296 |

**Examination of mice on days 4 and 6 of pregnancy**

*Tsg101*/*f/f* and *Tsg101*/*d/d* female mice (9 to 13-week-old) received 2.5 IU of eCG and hCG at 48 h intervals to promote mating. Immediately after hCG injection, they were bred with stud male mice. On the following morning, the formation of a vaginal plug was confirmed, and females with plugs were considered to be on day 1 of pregnancy. To examine implantation sites on day 6 of pregnancy, mice received a blue dye injection (1% Chicago blue B in phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and sacrificed 3 min later. When no implantation site was visible, uteri were flushed with M2 medium (M7167, Sigma-Aldrich). Some mice were sacrificed at 11 AM on day 4 of pregnancy to confirm the presence of embryos. One uterine horn was flushed with M2 media and the other was processed for histological analyses.

**Pseudopregnancy**

*Tsg101*/*f/f* and *Tsg101*/*d/d* female mice at 10 to 11-weeks of age received 2.5 IU of eCG and hCG at 48 h intervals. Immediately after hCG injection, the mice were bred with vasectomized ICR male mice. On the following morning, the formation of a vaginal plug was confirmed and females with plugs were considered to be on day 1 of pseudopregnancy. The uteri were collected from days 1, 4, or 6 of pseudopregnancy, and used for histological analysis and immunofluorescence staining. Uteri from day 4 pseudopregnant mice were used for uterine cell preparations.

**Histological analyses**

The uteri from pregnant or pseudopregnant mice were cut into small pieces and fixed in 4% paraformaldehyde (PFA) in PBS overnight. Using a tissue processor, samples were dehydrated and
embedded in paraffin. Sections (6–8 µm) were made using a microtome, placed onto a glass slide, and then subjected to hematoxylin-eosin (HE) staining. Slides were then examined using an upright microscope (Eclipse 80i, Nikon, Tokyo, Japan).

**Isolation of mouse uterine epithelial cells (UECs) and uterine stromal cells (USCs)**

Uteri from random cycling ICR (8-week-old), *Tsg101<sup>f/f</sup>* or *Tsg101<sup>d/d</sup>* mice received a subcutaneous injection of E<sub>2</sub> (100 ng/0.1 ml in sesame oil) 24 h before sacrifice to induce proliferation of UECs. Uteri pooled from 3 to 5 mice were cut into 3–4 mm pieces. Pancreatin (P3292; Sigma-Aldrich), dispase (17105–041; Gibco, Thermo Fisher Scientific), and collagenase (C1639; Sigma-Aldrich) were used to isolate uterine epithelial cells (UECs) and uterine stromal cells (USCs) as previously described [20]. Isolated UECs were filtered through a 70 µm nylon mesh filter (Corning, Sigma-Aldrich) to improve purity. UECs (2 × 10<sup>5</sup> cells) were grown on collagen-coated coverslips in a 6-well plate (Corning, Sigma-Aldrich) in DMEM/F12 (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (Lonza).

**Cell culture and necroptosis induction**

The L929 fibroblast cell line derived from mouse adipose tissue was obtained from the Korean Cell Line Bank (Seoul, Korea). L929 cells were cultured in DMEM media (11965–092, Gibco, Thermo Fisher Scientific) supplemented with 10% FBS (10099–141, Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (17-602E, Lonza, Basel, Switzerland). To induce necroptosis, UECs were treated with a mixture of 30 ng/mL TNF-α (PeproTech, Rocky Hill, NJ, USA), 10 µM Smac mimetic LCL-161 (R&D Systems, Minneapolis, MN, USA), and 20 µM ZVAD-FMK (R&D Systems) for 40 min [21]. Control cells were treated with 0.2% dimethyl sulfoxide (vehicle). L929 cell lysates were used as positive controls in western blotting.

**RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was extracted from pooled UECs and USCs isolated from several mice using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. RNA was treated with RQ RNase-free DNase (Promega, Madison, WI, USA) to remove any genomic DNA for 20 min at 25°C, followed by 10 min at 75°C to inactivate the DNase. RNA concentration and quality were assessed using a NanoDrop (ND-1000; Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from RNA using MMLV reverse transcriptase (BeamsBio, Seoul, Korea) and random hexamer primers (Invitrogen). Primers used for RT-PCR analyses are listed in Table 1. *Keratin 12 (Krt12)* and *desmin (Des)* were used as markers of the uterine epithelium and stroma, respectively [22].

**Western blotting**
Isolated UECs and USCIs were collected in RIPA buffer [10 mM Tris (pH 7.2), 150 mM NaCl, 0.1% Triton X-100, 5 mM ethylenediaminetetraacetic acid, 1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitors] and homogenized. The lysates were centrifuged at 12,600 × g for 15 min at 4°C and the supernatants collected. A bicinchoninic acid protein assay (Thermo Fisher Scientific) was performed to determine the concentration of the extract. The lysates were prepared in 4X sample buffer and boiled for 5 min. Samples were loaded onto SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk for 1 h and incubated overnight at 4°C with the primary antibodies shown in Table 2. The membranes were washed three times and then incubated with secondary antibodies (Table 2) at 25°C for 1 h. Chemiluminescence signals were detected using the West Save Detection Reagent A (Ab Frontier, Seoul, Korea) or West Femto kit (Thermo Fisher Scientific) and visualized with a LAS 4000 system (Fujifilm, Tokyo, Japan).
Table 2
Antibodies used in this study.

| Antibody        | Host  | Cat. no | Supplier          | Dilution | Application |
|-----------------|-------|---------|-------------------|----------|-------------|
| β-tubulin       | Rabbit| ab6046  | Abcam             | 1:2000   | WB          |
| RIPK1           | Mouse | 610459  | BD biosciences    | 1:500    | WB/IF       |
| pRIPK1          | Rabbit| 83613   | Cell signaling    | 1:500    | WB          |
| pRIPK1          | Rabbit| 31122   | Cell Signaling    | 1:1000   | IF          |
| RIPK3           | Rabbit| NBP1-77299 | Novus         | 1:500    | WB/IF       |
| pRIPK3          | Rabbit| 91702   | Cell signaling    | 1:500    | WB          |
| pRIPK3          | Rabbit| 57220   | Cell Signaling    | 1:1000   | IF          |
| MLKL            | Rat   | MABC604 | Merk              | 1:500    | WB/IF       |
| pMLKL           | Rabbit| ab196436| Abcam             | 1:500    | WB/IF       |
| E-cadherin      | Rabbit| 3195    | Cell signaling    | 1:200    | IF          |
| MUC1            | Rabbit| PA1-21077| ThermoFisher     | 1:200    | IF          |
| EEA1            | Rabbit| 2411    | Cell signaling    | 1:250    | IF          |
| Lamp1           | Rat   | NB100-77683 | Novus        | 1:125    | IF          |
| Desmin          | Goat  | Sc7559  | Santa Cruz        | 1:250    | IF          |
| Anti-rabbit IgG-HRP | Goat | SA002-500| GenDEPOT       | 1:10000  | WB          |
| Anti-mouse IgG-HRP | Goat | SA001-500| GenDEPOT       | 1:10000  | WB          |
| Anti-rat IgG-HRP | Goat  | 62-9520 | Invitrogen        | 1:10000  | WB          |
| Anti-rabbit IgG-Alexa Fluor 488 | Chick | A21441 | Invitrogen        | 1:250    | IF          |
| Anti-rat IgG-Alexa Fluor 488 | Donkey | A21208 | Invitrogen        | 1:250    | IF          |
| Anti-goat IgG-Alexa Fluor 488 | Rabbit | A21222 | Invitrogen        | 1:250    | IF          |
| Anti-mouse IgG-Alexa Fluor 488 | Donkey | A31571 | Invitrogen        | 1:250    | IF          |

Immunofluorescence staining and confocal microscopy

Cells were fixed in 4% PFA for 10 min and washed three times with PBS for 3 min each. Cells were then permeabilized with 0.25% Triton X-100 for 10 min and blocked with 2% bovine serum albumin (BSA) in
PBS for 1 h at 25°C. The cells were incubated with primary antibodies overnight at 4°C. After washing, the slides were incubated with secondary antibodies at 25°C for 1 h in the dark. DNA was counter-stained with TOPRO-3-iodide (Invitrogen).

For immunofluorescence staining of uterine sections, pieces from the uteri of Tsg101^f/f^ and Tsg101^d/d^ mice were fixed in 4% PFA in PBS overnight, followed by 30% sucrose solution overnight. The tissues were then frozen in optimal cutting temperature compound (Leica Biosystems, Wetzlar, Germany) with instant freezing aerosol. Sections (12 µm) were made using a cryostat (Leica Biosystems). The frozen sections were fixed in 4% PFA and permeabilized with 0.1% Tween-20 at 25°C for 20 min. After blocking with 2% BSA in PBS for 1 h at 25°C, the sections were incubated with primary antibodies overnight at 4°C. After washing, the slides were incubated with secondary antibodies at 25°C for 1 h in the dark. DNA was counter-stained with TOPRO-3-iodide. Images were obtained with a Zeiss LSM900 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) and analyzed with the ZEN Blue software (Carl Zeiss AG). Primary and secondary antibodies are shown in Table 2.

TUNEL assay

Apoptosis was analyzed using the DeadEnd Fluorometric terminal deoxynucleotidyl transferase-mediated dUUDP nick end labeling (TUNEL) assay kit (G3250; Promega). Paraffin sections of day 4 pseudopregnant uteri were deparaffinized in xylene, rehydrated through a graded series of ethanol, and washed with PBS. The sections were fixed in 4% PFA for 25 min and then permeabilized with 0.1% Triton X-100 for 5 min. The slides were equilibrated with equilibration buffer for 10 min and then incubated with recombinant terminal deoxynucleotidyl transferase incubation buffer at 37°C for 1 h and covered with plastic coverslips. Sections were incubated with 2X SCC buffer for 15 min and washed with PBS three times. The sections were counter-stained with TO-PRO-3-iodide (1:250 in PBS) for 15 min at 25°C in the dark and rinsed three times in PBS for 5 min each. The slides were mounted in antifade reagent (Invitrogen), examined under a Zeiss LSM900 confocal microscope and analyzed with the ZEN Blue software.

Live imaging during UEC culture

Isolated UECs were cultured in 60 mm dishes in culture medium. The cells were placed under a JuLI™ FL time-lapse microscope (JuLI-B004, NanoEntek, Seoul, Korea) in a CO₂ incubator. For activation of necroptosis, TNF-α (30 ng/mL), Smac mimetic LCL161 (10 µM), and z-VAD (20 µM) were added to the culture media as described above. UECs were stained with SYTOX™ Green Nucleic Acid Stain (S7020, Invitrogen) and imaged automatically at 1 h intervals.

RNA expression profiling

To compare the RNA expression profiles between Tsg101^f/f^ and Tsg101^d/d^ UECs, uteri from 3 Tsg101^f/f^ or 4 Tsg101^d/d^ mice were pooled and RNA extracted (n = 3 for each group). RNA quality was assessed using the 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). Total RNA (1 µg) obtained from the samples was used for RNA extraction with the MGIEasy RNA Directional Library Prep Kit (LAS, Gimpo, Gyeonggi-do, Korea) and processed for high-throughput sequencing using MGISEQ-2000. Volcano
plots for the expression-fold changes and p-values between the two selected samples were plotted by in-house R scripts. The top differentially expressed genes (DEGs) with ≥ 2-fold change (p ≤ 0.05) are shown as a heatmap, also drawn by an in-house R script. Significant changes in the biological processes based on Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and other functional gene sets were analyzed by g:Profiler version 0.6.7 [23].

**Statistical analysis**

Graphing and data analysis were performed using GraphPad Prism 5 software (https://www.graphpad.com/scientific-software/prism/) (Graph Pad Software, San Diego, CA, USA).

**Results**

**Generation of uterine epithelium-specific **$\text{Tsg101}$** deletion model**

The uterine epithelium-specific deletion of Tsg101 was achieved by crossing $\text{Tsg101}^{f/f}$ mice [8] with $\text{Ltfl}^{iCre}$ mice [19]. Deletion of $\text{Tsg101}$ in isolated UECs, but not in the uterine stromal cells (USCs), was confirmed by RT-PCR (Fig. 1A).

**Implantation failure in $\text{Tsg101}^{d/d}$ mice on day 6 of pregnancy**

$\text{Tsg101}^{d/d}$ female mice were bred with stud male mice for several months, however, they did not produce any pups. The status of pregnancy in the $\text{Tsg101}^{d/d}$ mice was examined on day 6 of pregnancy when implantation sites (IS) are generally visible. As shown in Fig. 1B, 6 out of 7 pregnant $\text{Tsg101}^{d/d}$ mice showed no IS, whereas control mice showed evenly spaced IS. The uterine flushings of $\text{Tsg101}^{d/d}$ uteri (4 out of 8 mice), showed unimplanted, zona-free blastocysts (Fig. 1B & D). $\text{Tsg101}^{d/d}$ uteri on day 6 showed variable thickness, as shown in Fig. 1B. Notably, the entire or portion of the uterus in some $\text{Tsg101}^{d/d}$ mice showed water imbibition (see Fig. 1B). Uterine histology of $\text{Tsg101}^{d/d}$ mice showed that the overall uterine architecture was normal, with all major cell types and glands visible (Fig. 1D). However, no luminal closure or decidualization was observed on day 6, suggesting that the implantation reaction was not initiated.

**Delayed embryonic development in $\text{Tsg101}^{d/d}$ uteri on day 4 of pregnancy**

We next examined if embryonic development proceeds normally to the blastocyst stage by day 4 of pregnancy in $\text{Tsg101}^{d/d}$ mice when the uterus is receptive to implantation. One uterine horn of $\text{Tsg101}^{f/f}$ and $\text{Tsg101}^{d/d}$ mice was flushed on day 4 of pregnancy, and the developmental stage of the embryos was monitored (Fig. 2, Table 3). At 11 AM on day 4, most embryos (81.25%) from $\text{Tsg101}^{f/f}$ mice were at the blastocyst stage, whereas only 43.3% of the embryos from the $\text{Tsg101}^{d/d}$ uteri were at the same stage (Table 3). These results show that embryonic development in $\text{Tsg101}^{d/d}$ mice is marginally delayed. Nonetheless, the presence of blastocysts on day 4 in $\text{Tsg101}^{d/d}$ mice, which failed to implant by day 6 of pregnancy was confirmed (Fig. 1B).
Table 3
The number of embryos in uterine flushings of day 4 pregnant mice.

| Genotype | No. of plug-positive mice | No. of mice with embryos | Total no. of embryos* | Total no. of blastocysts (%) | Total no. of morula (%) |
|----------|---------------------------|--------------------------|-----------------------|------------------------------|-------------------------|
| Tsg101^{f/f} | 8 | 6 | 32 | 26 (81.25) | 6 (18.75) |
| Tsg101^{d/d} | 9 | 6 | 30 | 13 (43.3) | 17 (56.7) |

* Mice received 2.5 IU of eCG followed by 2.5 IU hCG 48 h later and were individually caged with stud males to induce mating. The next morning, a mouse with a visible vaginal plug was designated to be on day 1 of pregnancy. Mice were sacrificed at 11 AM on day 4. One uterine horn from each mouse was flushed and the other horn was subjected to histological analysis.

The unflushed uterine horn was subjected to histological analysis (Fig. 2B). The overall uterine structures in Tsg101^{d/d} mice appeared normal, but the luminal epithelium seemed shorter (Fig. 2B, arrowheads and graph). Overall histological analyses suggested that the luminal epithelia of Tsg101^{d/d} uteri on day 4 of pregnancy was less developed than those of Tsg101^{f/f} uteri, displaying shortened heights. The average height of the luminal epithelium in Tsg101^{d/d} uteri was approximately 30 µm, about 20% lower than that of the Tsg101^{f/f} uteri (average 37 µm). This observation suggests that epithelial differentiation, which occurs during preparation for implantation, requires Tsg101 for structural integrity. E-cadherin and desmin, markers of the epithelium and stroma, respectively, showed an expected pattern of localization in Tsg101^{d/d} uteri, with E-cadherin in the uterine epithelium and desmin in the stroma (Fig. 2C).

**Molecular and cellular characteristics of UECs change in the absence of Tsg101**

In early pregnancy, the status of steroid hormones fluctuates depending on the day of pregnancy [14]. On day 1 of pregnancy, when preovulatory estrogen is dominant, the uterine epithelium proliferates extensively. On day 4, progesterone levels increase and a small amount of estrogen is secreted, driving epithelial differentiation and stromal proliferation in the uterus. On day 6 of pregnancy, when implantation has already taken place, the primary hormone modulating the uterus is progesterone secreted from corpora lutea [14]. To examine the status of the uterine epithelium under different hormonal profiles, we examined E-cadherin localization on days 1, 4, and 6 of pseudopregnancy in Tsg101^{f/f} and Tsg101^{d/d} mice. As shown in Fig. 3A, E-cadherin was localized to the uterine epithelium in both groups. Noticeably, a chunk of epithelial mass detached from the luminal epithelium was seen in pseudopregnant some day 1 Tsg101^{d/d} uteri. In day 4 pseudopregnant Tsg101^{d/d} uteri, some detached cells were also observed inside the lumen. The uterine epithelium on day 6 of pseudopregnancy showed E-cadherin localization. As indicated with arrowheads, the apical side of the luminal epithelium from day 4 pseudopregnant Tsg101^{d/d} mice appeared shorter than that of wild-type mice. We examined the localization of MUC1, a marker of apical side of the uterine epithelium [24]. In day 4 pseudopregnant Tsg101^{d/d} uteri, MUC1 showed a normal pattern in the apical surface (Fig. 3B, left panels). In another Tsg101^{d/d} mouse, the luminal epithelium was disorganized and collapsed (Fig. 3B) but retained E-
cadherin and MUC1 localization. Thus, the uterine epithelium retained its epithelial characteristics with intact marker expression but lost partial structural integrity. These results suggest that implantation failure in Tsg101<sup>d/d</sup> mice is associated with epithelial defects in the absence of Tsg101.

**Cultured Tsg101<sup>d/d</sup> UECs show a high rate of degeneration**

It has been previously shown that MEFs [8] and primary mammary epithelial cells [9] with Tsg101 knockdown show poor survival and various subcellular abnormalities. Thus, UECs isolated from Tsg101<sup>d/d</sup> uteri were cultured in vitro and cell survival was monitored. We observed that Tsg101<sup>d/d</sup> UECs began to degenerate within 18 h of culture (Fig. 4A). After 72 h, the number of remaining Tsg101<sup>d/d</sup> cells was much lower than Tsg101<sup>f/f</sup> UECs and stained positive for SYTOX Green stain, which stains cells with compromised plasma membranes (Fig. 4B).

The main function of Tsg101 is correlated with cytokinesis, endosomal trafficking, and the formation of the late endosomal structures called MVBs [25]. Therefore, we examined whether endolysosomal structures in Tsg101<sup>d/d</sup> UECs were normal. Localization of early endosome antigen 1 (EEA1) and lysosome-associated membrane protein 1 (LAMP1) was examined by immunofluorescence staining (Fig. 4C). As shown in Fig. 4C, both EEA1 and LAMP1 exhibited puncta-like localization mostly in the perinuclear region, which did not differ between the Tsg101<sup>f/f</sup> and Tsg101<sup>d/d</sup> UECs. These results collectively suggest that cultured UECs tend to degenerate in the absence of Tsg101 without noticeable endolysosomal defects. Thus, increased UEC death could be associated with implantation failure in Tsg101<sup>d/d</sup> mice.

**Expression of necroptosis factors in UECs**

Another role for Tsg101 and other ESCRT factors has recently been suggested which involves counteracting necroptotic cell death [13]. Necroptosis can be induced by various external and internal stimuli, and the resulting plasma membrane breach is generally mediated by the RIPK1-RIPK3-MLKL pathway [11]. Whether UECs express such necroptosis effectors has not been reported. We examined the expression of these factors by RT-PCR and western blotting (Fig. 5A & B). We confirmed the expression of Ripk1, Ripk3, and Mlkl in isolated UECs and USC.

In various cell types, RIPK1, RIPK3, and MLKL respond to necroptosis-inducing signals and undergo phosphorylation [12]. In L929 mouse fibroblast cells, combined treatment with TNFα (T), LCL161 (S, a Smac mimetic), and zVAD-fmk (Z, an apoptosis inhibitor) induced the phosphorylation of these three factors [21]. Using TSZ-treated L929 cells as a positive control, we examined the status of RIPK1, RIPK3, MLKL, and their phosphorylated forms by western blotting. As shown in Fig. 5B, all three factors were present in both UECs and USC. Since their phosphorylated forms were also detected in UECs and USC without external stimulation, it is possible that a basal level of necroptosis may be operative in these cells. Immunofluorescence staining of these factors mostly showed a scattered punctate pattern in the
cytoplasm. As for phosphorylated MLKL (pMLKL), it was localized in some UECs in the plasma membrane, which is known to occur upon activation of necroptosis (Fig. 5C, white arrowhead) [26].

We then tested whether UECs respond to exogenous necroptosis-inducing signals. UECs were treated with TSZ for 24 h in the presence of the SYTOX Green live dye. As shown in Fig. 5D, TSZ treatment dramatically increased SYTOX Green-positive UEC cells. Thus, UECs are equipped with necroptosis effectors and can respond to necroptosis-inducing exogenous signals.

**Cell death in Tsg101<sup>d/d</sup> UECs**

The final executor of necroptosis is pMLKL, which induces permeabilization of the plasma membrane [12]. Whether the luminal epithelium expresses active pMLKL during pregnancy is unknown. We examined whether pMLKL is localized to the luminal epithelium on day 4 of pseudopregnancy (Fig. 6A). In the Tsg101<sup>f/f</sup> uteri, pMLKL showed a punctate localization in a portion of the apical surface of the luminal epithelium (Fig. 6A). In the Tsg101<sup>d/d</sup> uteri with shortened luminal epithelium, the pMLKL signal was not as distinct as in the Tsg101<sup>f/f</sup> uteri.

To investigate whether detachment of cells in the Tsg101<sup>d/d</sup> uterus is associated with apoptosis, we performed TUNEL staining on day 4 pseudopregnant uterine sections (Fig. 6B). Again, we found cells detached from the luminal epithelium and a higher number of TUNEL-positive cells in the subepithelial stromal regions in the Tsg101<sup>d/d</sup> uterus.

**mRNA expression landscape in the Tsg101<sup>f/f</sup> and Tsg101<sup>d/d</sup> UECs**

To compare the overall mRNA expression landscape between the Tsg101<sup>f/f</sup> and Tsg101<sup>d/d</sup> UECs, we performed mRNA expression profiling. To avoid mRNA contamination from the embryos, UECs from day 4 pseudopregnant mice were used. These samples were subjected to mRNA expression profiling. Heatmaps of the top 50 differentially expressed genes (DEGs) are shown in Fig. 7A. Remarkably, genes upregulated in the Tsg101<sup>d/d</sup> UECs exhibited high variation between the samples (Fig. 7A, left panel), whereas genes downregulated in Tsg101<sup>d/d</sup> UECs showed a more consistent trend (Fig. 7A, right panel). In total, 1284 genes were differentially expressed between Tsg101<sup>f/f</sup> and Tsg101<sup>d/d</sup> UECs. Of these DEGs, 734 genes were upregulated, whereas 550 genes were downregulated in Tsg101<sup>d/d</sup>. Histological examination of day 4 pseudopregnant uteri from some Tsg101<sup>d/d</sup> mice used in this experiment showed patches of cells in the lumen (Fig. 7B). One of the downregulated gene in Tsg101<sup>d/d</sup> UEC, *Aquaporin 8* (*Aqp8*), indeed were shown decreased in these samples (Fig. 7C). Fluid accumulation we had observed in several Tsg101<sup>d/d</sup> uteri (Fig. 1B) may be associated with aberrant expression of water channel genes [27].

GO term enrichment and KEGG pathway analyses were used to identify key genes and pathways operative in Tsg101<sup>f/f</sup> and Tsg101<sup>d/d</sup> UECs (Fig. 8). In terms of DEGs in GO term enrichment analysis for the biological process (Fig. 8A), several gene classes associated with immune functions were upregulated in Tsg101<sup>d/d</sup> UECs. KEGG pathway analysis of the DEGs showed clustering of several
signaling pathways, such as cell adhesion molecules and cytokine-cytokine receptor interaction (Fig. 8B). Together, these results suggest that various cellular functions were affected in the uterine epithelium in the absence of Tsg101.

Discussion

Tsg101 was initially cloned as a candidate tumor suppressor gene in mice [28]. While several reports suggest a role for this gene in tumor suppression [7], other complex and fundamental roles for Tsg101 in cells have been uncovered, ranging from endolysosomal maturation, cytokinesis, cell proliferation, and survival [7]. As the deletion of Tsg101 in mice is lethal early in development [8], the biological functions of Tsg101 have been investigated in several tissue-specific Tsg101 deletion mouse models [7]. In mammary epithelial cells, cardiomyocytes, and oligodendroglia, Tsg101 deletion leads to cell death accompanied by apoptosis, vacuolation, and other subcellular changes [29–31].

Our study shows that Tsg101 plays a crucial role in maintaining the integrity of the uterine epithelium during embryo implantation. The Ltf-iCre mice achieved efficient and specific deletion of the floxed genes in the uterine epithelium at approximately 2 months of age [19]. All mice used in our experiments were between 8–11-weeks of age. By this time, all uterine structures have formed and sexual maturation is complete. Thus, the subfertility phenotype observed in Tsg101<sup>d/d</sup> mice is irrelevant to anatomical and endocrinological abnormalities. Our results show that Tsg101 is required in the uterine epithelium to initiate embryo implantation (Fig. 1). The presence of well-formed, zona-free blastocysts in day 6 pregnant Tsg101<sup>d/d</sup> uteri suggests that the luminal epithelium is unable to support implantation.

On day 4 of pregnancy, Tsg101<sup>d/d</sup> uteri contained preimplantation embryos at the morula and blastocyst stages (Fig. 2A). It is unclear whether Tsg101 deletion in the uterus is directly or indirectly associated with delayed embryonic development on day 6 (Table 3). On this day, the uterus is under the influence of progesterone and estrogen, both of which influence dynamic cellular and molecular changes required for implantation [32]. In some Tsg101<sup>d/d</sup> mice at 10–11-weeks of age, the luminal epithelia showed a disorganized pattern and height shortening (Fig. 3A, arrowheads). Patches of cells were also found in the uterine lumen of some Tsg101<sup>d/d</sup> mice. While E-cadherin and MUC-1, markers of the epithelium, exhibited normal localization in the epithelium, these markers helped identify the collapsed epithelial structure in some Tsg101<sup>d/d</sup> mice (Fig. 3B). When UECs were isolated and cultured in vitro, Tsg101<sup>d/d</sup> UECs began to show signs of degeneration around 24 h with the emergence of clustered cell patches (Fig. 4), which are uncharacteristic of epithelial cells. Tsg101<sup>d/d</sup> UECs also showed increased cell permeabilization, as was previously observed in certain ESCRT factor-depleted cells [13]. It was previously shown that Tsg101-depleted MEFs showed enlarged lysosomal structures, along with other complex cellular changes [9]. In the UECs, we did not observe a similar pattern. In vertebrate epithelial cells, ESCRT factors have been implicated in the maintenance of polarity [3]. Considering that MEFs are of mesenchymal origin, Tsg101 and other ESCRT factors may play distinct roles depending on the cell type.
Necroptosis can be initiated by various stimuli, such as death ligands and bacterial toxins, but can also be induced during normal physiological conditions and aging [12, 33]. Here, we show for the first time that UECs express the major necroptosis effectors, RIPK1, RIPK3, and MLKL, and their phosphorylated forms (Fig. 5C, arrowhead). pMLKL localization to the cell edge (Fig. 5C) suggests that UECs show active necroptosis [13, 34]. When TSZ was used to induce necroptosis [21], UECs showed a dramatic increase in SYTOX Green staining, which further supports the notion that UECs respond to external stimuli and activate necroptosis. Consistent with this finding, the Tsg101<sup>f/f</sup> uterine epithelium on day 4 of pregnancy showed a distinct punctate pattern of pMLKL localization on the epithelial edge (Fig. 6A). The presence of pMLKL implies active necroptosis involving the cell membrane. Thus, our results suggest that UECs harbor a functional necroptotic machinery. The degeneration of cultured Tsg101<sup>d/d</sup> UECs and disintegration of the uterine epithelium in Tsg101<sup>d/d</sup> uteri may be associated with a failure to counteract the necroptotic activation that occurs as a part of the normal physiology of these cells.

Finally, we compared RNA expression profiles between the Tsg101<sup>f/f</sup> and Tsg101<sup>d/d</sup> UECs (Fig. 7&8), but the RNA expression among the different Tsg101<sup>d/d</sup> UEC samples tended to show a high variation. Such high variation precluded us from pinpointing target pathways and genes associated with Tsg101 in the uterine epithelium. This could be partially due to epithelial disintegration in some Tsg101<sup>d/d</sup> mice (Fig. 3B), highlighting the importance of Tsg101 in maintaining uterine tissue architecture.

**Conclusions**

To date, the role of necroptosis and ESCRT factors in regulating uterine physiology and embryo implantation is not known. We confirm, for the first time, the presence of necroptosis effectors in UECs. UECs also responded to an exogenous necroptosis-inducing stimulus, involving a combination of TNFa, Smac mimetics, and an apoptosis inhibitor, and showed increased membrane permeabilization. However, Tsg101<sup>d/d</sup> UECs degenerated in vitro, even in the absence of such external stimuli. Thus, it is reasonable to assume that Tsg101 is required to sustain the survival of cultured UECs. Since UECs showed a tendency to disintegrate within Tsg101<sup>d/d</sup> uteri in vivo, it would be interesting to investigate how the tissue architecture of the uterus is maintained in older Tsg101<sup>d/d</sup> mice. Whether the uterine epithelium degenerates completely or cells of a different origin replace the epithelium in the Tsg101<sup>d/d</sup> uteri, requires further investigation. Our model can be further applied to study cell-to-cell interactions during uterine regeneration. The regulation of necroptosis in UECs and its role in uterine physiology warrants further investigation. How this cell death mechanism is related to inflammation-associated uterine pathology is another relevant topic that needs to be pursued in the future.

**Abbreviations**

ESCRT: Endosomal sorting complex required for transport

Tsg101: Tumor susceptibility gene 101
eCG: equine chorionic gonadotropin

hCG: human chorionic gonadotropin

MLKL: Mixed lineage kinase-like protein

RIPK1: Receptor interacting protein kinase 1

RIPK3: Receptor interacting protein kinase 3

Declarations

Ethics approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of supporting data

Data supporting the findings are presented within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

H.B., S.K., H.S., and H.J.L. devised the study; H.B., S.K., and H.S. performed the experiments; H.B., S.K., H.S., K-U. W. and H.J.L. analyzed the data; H.B. and H.J.L. wrote the manuscript with input from all authors.

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