Cell Reports

Supplemental Information

Entosis Allows Timely Elimination of the Luminal Epithelial Barrier for Embryo Implantation

Yingju Li, Xiaofei Sun, and Sudhansu K. Dey

Supplemental figures

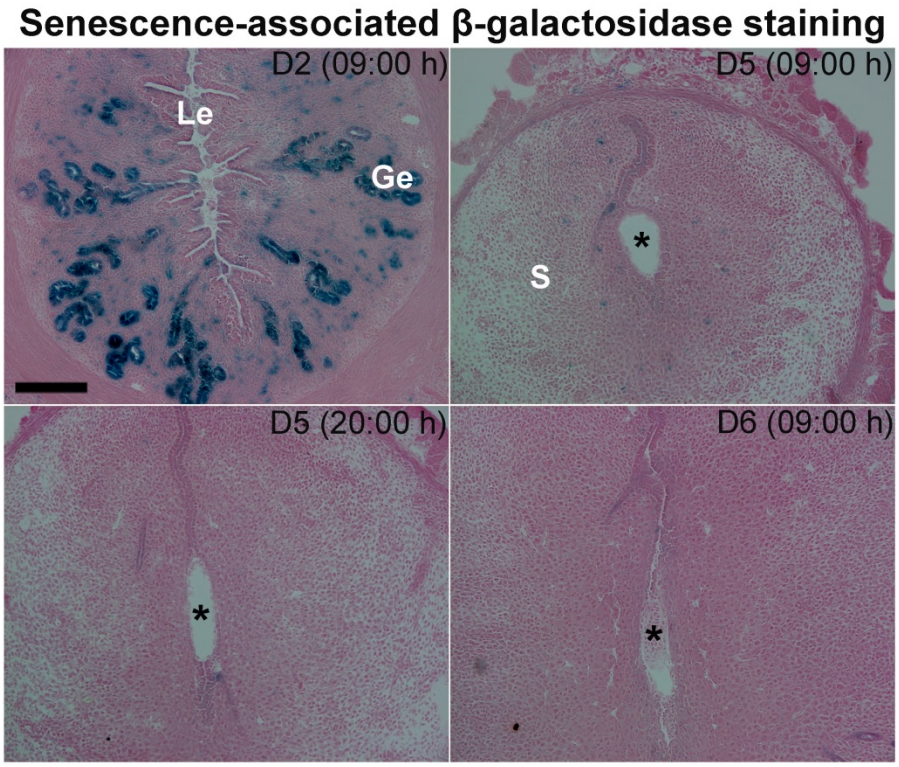

Figure S1. **Senescence-associated β-galactosidase (β-gal) activity is absent in LE cells in the morning or evening of day 5 of pregnancy, related to Figure 2A.** The section of a uterus on day 2 served as a positive control. Le, luminal epithelium; Ge, glandular epithelium; S, stroma. Asterisks indicate the position of the embryo. Bar, 200 μm.

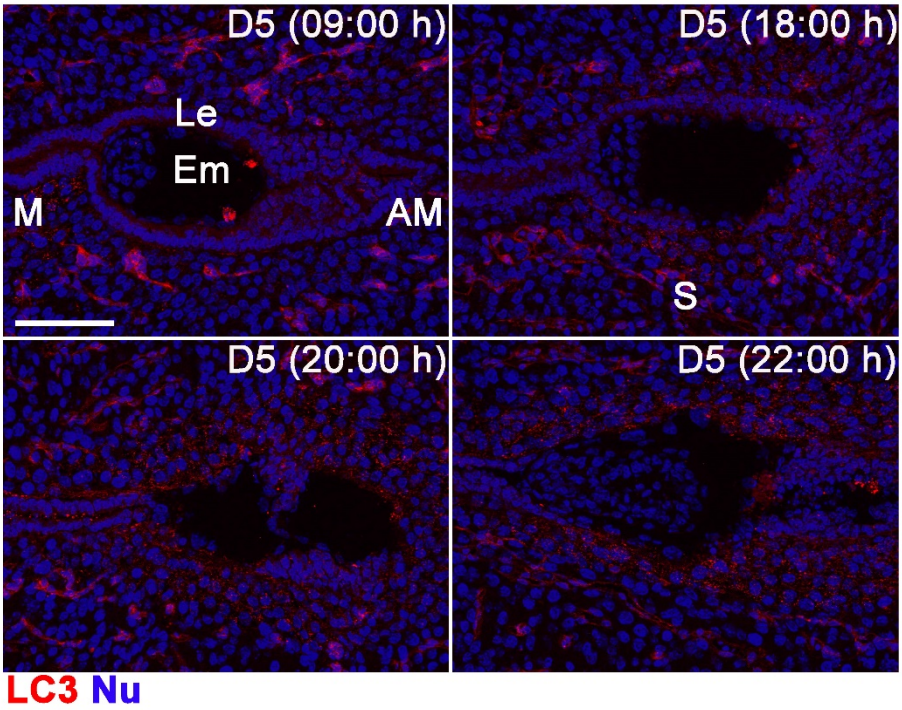

Figure S2. **Autophagy signals are absent in LE cells on day 5, related to Figure 2A.** Two embryonic trophoblast (Tr) cells showed positive LC3 signals at 09:00 h on day 5, but the uterine LE cells are negative in the morning or evening of day 5 and 6 of pregnancy. Le, luminal epithelium; S, stroma; Em, embryo; M, mesometrial pole; AM, antimesometrial pole. Bar, 100 μm.

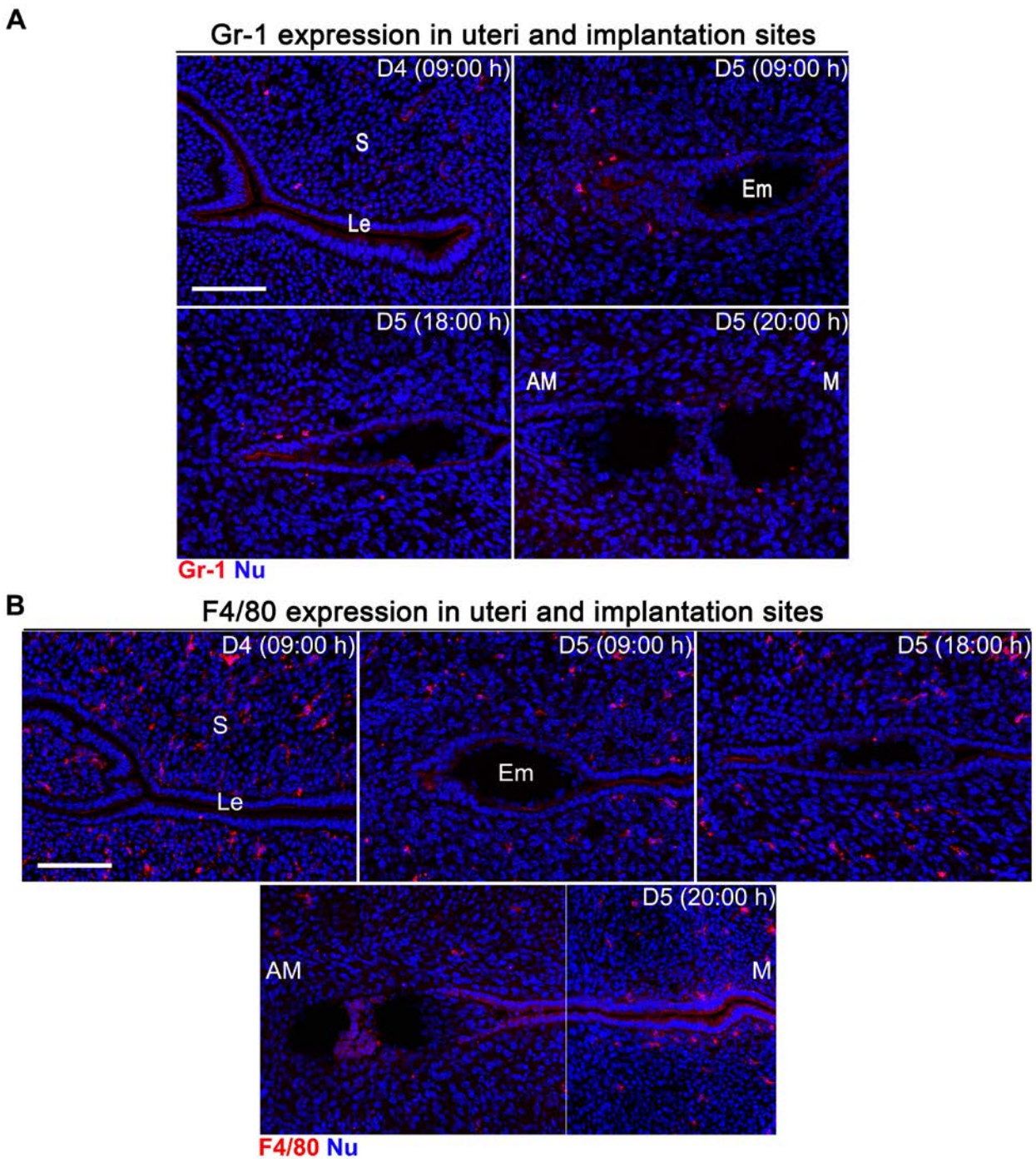

Figure S3. **Neutrophils and macrophages are not responsible for LE cell phagocytosis, related to Figure 3.** Gr-1 (neutrophil) and F4/80 (macrophage) positive cells were absent at the implantation sites from 09:00 h of day 5. Two images of adjacent fields from a section of the implantation site on day 5 (20:00 h) placed together are shown in panel B. Le, luminal epithelium; S, stroma; Em, embryo; M, mesometrial pole; AM, antimesometrial pole. Bars, 100 µm.

Supplemental movie 1. **Internalization of a uterine epithelial cell by a TS cell in suspension, related to Figure 4A**. Z series of confocal microscopic analysis demonstrating a TS cell (Red CellTracker) engulfing an epithelial cell (Green CellTracker).

Supplemental movie 2. **Blastocyst trophoblasts engulf uterine epithelial cells in suspension, related to Figure 4C.** A 3-D view of confocal microscopic analysis demonstrating a reporter blastocyst (Rosa-Tomato) engulfing epithelial cells (Green CellTracker).

Supplemental experimental procedures

Animals

Female mice were housed with fertile males in the evening and checked for vaginal plug next morning at 09:00 h to confirm successful mating (vaginal plug=day 1 of pregnancy). Plug-positive females were killed as various times as indicated in the text; implantation sites were separated, flash-frozen or fixed for further analyses. To collect reporter blastocysts, uteri were collected from [*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$]Tomato females and mated with wild-type (WT) males on day 4 of pregnancy (Muzumdar et al., 2007). Blastocysts were obtained by flushing the uteri and cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS). To induce pseudopregnancy, WT female mice were mated with vasectomized males. To induce implantation-like responses, 10 µL of sesame oil were infused into one uterine lumen. They were killed at different time points as indicated and analyzed. For in vivo experiments with a ROCK inhibitor Fasudil, pregnant females were injected intraperitoneally with Fasudil (0.75 mg/100 µl/mouse) at 09:00 h on day 5 of pregnancy and their uteri were collected at 20:00 h on day 5. All protocols for the present study were reviewed and approved by the Cincinnati Children's Research Foundation's Institutional Animal Care and Use Committee. Mice were provided with autoclaved rodent Lab Diet 5010 (Purina) and UV light-sterilized RO/DI constant circulation water *ad libitum*.

Cell Tracker assays

Monolayer cultures of primary epithelial and TS cells were stained with Cell-Trackers Green and Red (Invitrogen) for 30 min at 37°C in the absence of serum (Overholtzer et al., 2007). Stained red and green cells were washed twice in PBS, trypsinized to single cell suspensions, and mixed 1:1 on polyhema-coated plates at 1-200,000 cells per ml of culture media. Six hours after incubation, suspended cells were stained with Hoechst 33342 (5 µg/ml for 15 min at 37°C), collected, washed in PBS, and fixed in 2% formalin for 10 min at room temperature. Fixed cells were washed in PBS and a portion of fixed cells was immunostained by antibody for β-catenin (Santa Cruz). The cells were then embedded in 0.2% agar. Agar pellets with embedded cells were minced with a razorblade and mounted onto glass slides with Fluoromount-G (Southern Biotech). In

cell tracker assays using mouse blastocysts and primary epithelial cells, only epithelial cells were stained as described above. Blastocysts were co-incubated with stained epithelial cells at 100,000 cells per ml of culture media. Six hours after incubation, blastocysts were collected, stained with Hoechst 33342 (5 µg/ml for 15min at 37°C), washed in PBS, and fixed in 2% formalin for 10 min at room temperature. Fixed blastocysts were washed in PBS and embedded in 0.2% agar.

SA-β-gal staining.

Staining of SA-β-gal activity was performed as described previously (Hirota et al., 2010). Sections of tissues from different days of pregnancy were processed on the same slide. In brief, frozen sections were fixed in 0.5% glutaraldehyde in PBS and stained for 6 hours in PBS (pH 5.5) containing 1mM MgCl2, 1mg/ml X-gal, and 5mM each of potassium ferricyanide and potassium ferrocyanide. Sections were counterstained with eosin.

Histological analysis and Immunostaining.

Formalin-fixed paraffin embedded uterine sections (5 µm) were stained with hematoxylin and eosin for histological analysis or immunostained for E-Cadherin (Cell signaling). After deparaffinization and hydration, sections were subjected to antigen retrieval by autoclaving in 10 mM sodium citrate solution (pH=6.0) for 10 min. A diaminobenzidine (DAB, Sigma) solution was used to visualize antigens. Sections were counter-stained with hematoxylin (Hirota et al., 2010). In immunofluorescence studies, formalin-fixed frozen sections (12 µm) were blocked with 5% BSA in PBS and immunostained by antibodies for E-Cadherin (Cell signaling), β-catenin (Santa Cruz), CK8 (the Developmental Studies Hybridoma Bank, Iowa), ZO-1 (Life technology), Cleaved Caspase 3 (Cell signaling), GR-1 (AbD Serotec), F4/80 (AbD Serotec), LAMP1 (the Developmental Studies Hybridoma Bank, Iowa) and LC3 (Abcam). Signals were visualized by secondary antibody conjugated with Cy2 or Cy3 fluorophore (Jackson Immunoresearch). Sections were counter-stained with Hoechst 33342 (2 µg/ml, Life technology).

Supplemental References

Hirota, Y., Daikoku, T., Tranguch, S., Xie, H., Bradshaw, H.B., and Dey, S.K. (2010). Uterine-specific p53 deficiency confers premature uterine senescence and promotes preterm birth in mice. The Journal of clinical investigation *120*, 803-815.

Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. Genesis *45*, 593-605.

Overholtzer, M., Mailleux, A.A., Mouneimne, G., Normand, G., Schnitt, S.J., King, R.W., Cibas, E.S., and Brugge, J.S. (2007). A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. Cell *131*, 966-979.