A tissue-engineered uterus supports live births in rabbits

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Bioengineered uterine tissue could provide a treatment option for women with uterine factor infertility. In large animal models, reconstruction of the uterus has been demonstrated only with xenogenic tissue grafts. Here we use biodegradable polymer scaffolds seeded with autologous cells to restore uterine structure and function in rabbits. Rabbits underwent a subtotal uterine excision and were reconstructed with autologous cell-seeded constructs, with nonseeded scaffolds or by suturing. At 6 months postimplantation, only the cell-seeded engineered uteri developed native tissue-like structures, including organized luminal/glandular epithelium, stroma, vascularized mucosa and two-layered myometrium. Only rabbits with cell-seeded constructs had normal pregnancies (four in ten) in the reconstructed segment of the uterus and supported fetal development to term and live birth. With further development, this approach may provide a regenerative medicine solution to uterine factor infertility.

The uterus supports many complex biological functions that are essential for mammalian reproduction. Factors adversely affecting uterine integrity, such as congenital anomalies or acquired diseases, may compromise a woman's ability to conceive or to carry a viable fetus to term. Approximately 6% of women undergoing infertility treatments have a dysfunctional uterus. Allogeneic uterine transplantation has shown promise as a treatment for permanent uterine infertility, although it requires donors and the use of antirejection therapies. Since 2014, live births have been reported in women who received transplanted uteri. Regenerative medicine and tissue engineering technologies have emerged as an attractive option for overcoming donor organ shortages and other limitations of allogeneic transplantation. Creating tissue and organ substitutes from a patient's own cells combined with biomaterials minimizes the risks of immunological rejection and disease transmission. This approach has enabled the implantation of bioengineered bladders, blood vessels, urethras and vaginas in human patients.

Previous work on regeneration of the uterus has proved successful only for small defects that do not have the critical size needed for clinical translation. In rodent models, small uterine defects regenerated when repaired with a variety of biomaterials alone, without the use of cells, such as nondegradable synthetic polymer scaffolds, biodegradable synthetic polymer scaffolds, naturally derived scaffolds and even scar tissue. However, attempts at regenerating larger uterine defects using these strategies have failed. Moreover, rodent organs, including the uterus, have an inherent regenerative capacity. Thus, results obtained in rodent models may not translate to larger animal models and humans. Only two published studies have explored uterine regeneration in an animal model larger than rats. One study used human umbilical vein grafts as a segmental graft in rabbits and reported grafts with epithelial layer reorganization but minimal myometrial tissue ingrowth. In the second study, porcine small-intestinal submucosa acellular grafts ranging from 0.5 to 2 cm in length by 0.7–0.8 cm were engrafted in rabbits, and the success of the study was limited as the graft size increased. It was noted that grafts 1 cm or longer collapsed, resulting in total architectural disruption.

Findings in other rabbit studies on reconstruction of hollow structures, such as tubular urethras, also demonstrated abnormal regeneration when using 1-cm acellular matrices alone, but if cells were seeded on the same 1-cm matrices, normal regeneration would occur. Acellular matrices covering defect areas allow cells from the normal native tissue edge to move across the matrix, creating new tissue. The maximum defect distance suitable for normal urethral tissue formation in rabbits using acellular grafts that rely on the surrounding native tissue cells for regeneration is 0.5 cm (ref. 1). If the biomaterial distance is greater than 0.5 cm from any native edge, there is still some cell migration from the native tissue, but fibroblast deposition takes over, leading to scar formation. This also explains, in part, why rodent animal models are not ideal for regeneration studies, as the size of the defects created in most instances may not be clinically meaningful, and why previous rabbit studies with larger defects failed. Because of the inherent regenerative capacity of small segments of tissue in rodent models, larger animal models, where one can replace critical-size defects with engineered constructs, are important in highlighting potentially human clinically relevant regeneration, both scientifically and for advancing these technologies to humans. Rabbits have long been used in reproductive biology research and are ideal for uterine tissue regeneration studies as they have a relatively large uterus compared with other laboratory animals, with two separated functional uterine horns and cervices, each with a capacity to carry a pregnancy.

Our strategy to bioengineer functional tissues using autologous primary cells seeded onto biodegradable scaffolds has been effectively explored in preclinical studies, and applied successfully in human patients to restore function in tubular organs such as the urethra, as well as in hollow nontubular organs, such as the bladder and vagina. We now follow the same approach to engineer the uterus, a more complex organ with higher functional requirements involving support of embryo implantation and fetal development. We used biodegradable polymer scaffolds, 6–8 cm in length and 2.5 cm in width, composed of poly-dl-lactide-coglycolide (PLGA)-coated polyglycolic acid (PGA) seeded with primary uterine-derived cells. We have previously used PGA in human patients combined with PLGA to engineer urethras, and combined with collagen to engineer bladders. PGA and PLGA polymer biomaterials have been used in tissue engineering due to their unique properties, including high porosity and interconnectivity between...
pores, high surface area-to-volume ratio, versatility of chemistry, tunable mechanical properties and biocompatibility.

We describe the in vitro fabrication of engineered uterine tissue and its development in a rabbit model of subtotal uterine reconstruction. Over time, the engineered uterine tissue formed patent cavities, organized cellular and anatomical structures inside the endometrium and myometrium compartments. Our reproductive studies showed that autologous tissue-engineered constructs supported late-stage pregnancies and viable live births.

Results
Rabbits underwent full excision of one uterine horn and a subtotal excision of the remaining uterine horn, and were divided into three experimental groups, with two groups receiving either a tissue-engineered scaffold or a nonseeded scaffold and a third group where the remaining uterine edges were sutured together. Rabbits from all three experimental groups tolerated the procedures well and subsequently underwent reproductive studies and tissue analyses.

Characterization of cultured primary uterine cell. Cells used for seeding the scaffolds were collected from a full-thickness biopsy of the excised uterine horn. Cells were characterized using immunocytochemistry and flow cytometry. The inner layer of the full-thickness uterine biopsies (Supplementary Fig. 2a) yielded cuboidal and elongated cells (Supplementary Fig. 2b) that expressed epithelial and stromal markers cytokeratin AE1/AE3 (1:50, ab28028, Abcam; Supplementary Fig. 2c) and vimentin (1:100, ab28028, Abcam; Supplementary Fig. 2d), respectively. The outer layer of the uterine biopsies yielded spindle shaped smooth muscle-like cells (Supplementary Fig. 2e) that were positive for smooth muscle myosin heavy chain 11 (MHC, 1:250 ab683; Abcam), calponin (1:100, C2687; Sigma-Aldrich), and smooth muscle alpha-actin (α-SMA, 1:40, ab18147; Abcam; Supplementary Fig. 2f–h). Flow cytometric analyses of third-passage cultured cells showed that a high percentage of the endometrium-derived samples expressed the epithelial cell marker CD9 (refs. 28,29) (1:100, CBL162P, Millipore; 78%) and stromal marker vimentin (1:100, 96%), while myometrium-derived cultures expressed αSMA (1:100, 96%) and calponin (1:100, 90%) (Supplementary Figs. 2i–l and Supplementary Fig. 2m for flow cytometry gating). These phenotypes and cell marker profiles are consistent with native endometrial and myometrial cells and aligned with previous reports that primary uterine cells, without fibroblast overgrowth, are stable in culture systems for over ten passages.

Fabrication of the uterine constructs. PGA/PLGA scaffolds were tailor-made in semicircular shapes (Fig. 1a). Scanning electron microscopy (SEM) micrographs of representative samples of the synthetic polymer scaffolds depicted a three-dimensionally interconnected porous network (Fig. 1b). Ten million cells per cm² collected from the myometrium-derived cultures were seeded on the outer layer of the scaffold and ten million endometrium-derived cells per cm² were successfully seeded on the inside of the scaffold using a sequential seeding method. SEM analysis of the PGA/PLGA seeded scaffolds showed uniform cell attachment throughout the length and thickness of constructs during the in vitro incubation period (Fig. 1c).

Implantation of the uterine constructs. At the time of the experimental implantation procedure, the rabbits had a subtotal semicircular excision of their remaining uterine horn and received a cell-seeded construct, a nonseeded scaffold or had the defect repaired by suturing the remaining edges. Titanium clips were used to delineate the margin between the native tissue and the area of scaffold implantation. An additional normal control group underwent a sham laparotomy. The surgical procedures (Fig. 1c,f) were uneventful and no great postoperative complications were observed during the study.
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into the cervical canal under fluoroscopic observation to investigate the morphology of the uterine cavities. Serial hysterograms taken at 6 months showed that the tissue-engineered group had patent cavities with a preserved uterine configuration (Supplementary Fig. 3a). The nonseeded scaffold group had multiple filling defects and marked strictures along the lumen of the implanted uterine horn (Supplementary Fig. 3b). The subtotal excision-only controls had severe proximal luminal blockage with incomplete contrast filling of the cavity (Supplementary Fig. 3c). The normal control uterine horns showed patent cavities that resembled the tissue-engineered uteri group (Supplementary Fig. 3d).

In vivo analyses of uterine constructs. Thirty-six of the 78 animals were killed at 1, 3 or 6 months after the experimental uterine surgeries for analyses (n = 3 per experimental group per time point). During necropsy, the reproductive tract was inspected for gross abnormalities. The uterine surgical sites were identified in all experimental animals. Specimens were retrieved from the engrafted area, identified by the presence of marking sutures and titanium clips and then submitted for histological analysis.

Gross findings. Gross macroscopic observations at 1 month after surgery showed mild adhesions between the uterus and adjacent organs (bladder and intestine), as well as a partially degraded biomaterial in the scaffold groups. Animals in the subtotal excision-only group had anastomotic strictures and hydrometro in 67% of the retrieved uterine horns. At 3 and 6 months, the polymer scaffold material was not visually present in the engineered uterine group or in the nonseeded scaffold group. At 6 months, the subtotal excision-only group had areas of exposed endometrium and fibrotic scars along the surgical site. Before euthanasia contrast medium was instilled into the cervical canal under fluoroscopic observation to investigate the morphology of the uterine cavities. Serial hysterograms taken at 6 months showed that the tissue-engineered group had patent cavities with a preserved uterine configuration (Supplementary Fig. 3a). The nonseeded scaffold group had multiple filling defects and marked strictures along the lumen of the implanted uterine horn (Supplementary Fig. 3b). The subtotal excision-only controls had severe proximal luminal blockage with incomplete contrast filling of the cavity (Supplementary Fig. 3c). The normal control uterine horns showed patent cavities that resembled the tissue-engineered uteri group (Supplementary Fig. 3d).

Immuno-histological/molecular findings. Tissue cross-sections were stained with Masson’s trichrome and used to calculate the relative collagen and smooth muscle content in samples retrieved at 1, 3 and 6 months. At 1 month postimplantation, we observed that the scaffold material had partially degraded histologically and maintained the luminal cavity structure in animals receiving tissue-engineered uterine constructs and nonseeded scaffolds (Fig. 2a,b). In contrast, the subtotal excision-only controls showed abundant granulomatous tissue formation and complete luminal stenosis (Fig. 2c). At
the anastomosis site, we observed initial tissue growth in all experimental groups, but the tissue-engineered uteri group had greater full-thickness cellular coverage surrounding the polymer fibers (Fig. 2a middle, bottom) with less collagen content than nonseeded scaffold group and subtotal excision-only controls (37 ± 5%, 68 ± 4% and 64 ± 4%, respectively; P < 0.01) (Fig. 2m). Three months after surgery, the scaffold material underwent complete biodegradation. The tissue-engineered uteri group had developed an epithelial lining, distinct stroma with glandular structures (Fig. 2e middle) and smooth muscle bundles in the outer layer (Fig. 2f bottom). The nonseeded scaffold group had epithelial coverage, partially formed stromal layer (Fig. 2f middle), scarce smooth muscle fibers (Fig. 2f bottom) and greater collagen content compared to the tissue-engineered group (61 ± 5% versus 39 ± 3%, P < 0.01) and normal controls (61 ± 5% versus 25 ± 2%, P < 0.001) (Fig. 2m). The subtotal excision-only controls had an incomplete development of the endometrial and myometrial

Fig. 3 | Histomorphological analysis of the uterine mucosa at 6 months postimplantation. a, e, g, H&E cross-sections of uterine horns. Scale bars, 500 µm on the left and 100 µm on the right. b, d, f, h, Immunostaining for CD31 indicates positive endothelial cells in the capillaries and lining of mature blood vessels. Scale bars, 50 µm. i, Analysis of the average endometrial thickness measured at the points of greatest perpendicular depth under a magnification of ×100 showed that the tissue-engineered uteri group formed thicker inner layer than the nonseeded scaffold group and subtotal excision-only controls. j, Quantitative analysis of the average number of endometrial glands per field using a ×20 objective showed that the endometrial gland density was comparable between the tissue-engineered uteri group and normal controls. k, Quantitative analysis of the average number of microvessels per field under a magnification of ×200 showed greater endometrium neovascularization in tissue-engineered uteri group than the nonseeded group and subtotal excision-only group. Black arrows indicate the interface between the native uterine tissue and engrafted site. Yellow arrowheads indicate endometrial glands; red arrowheads indicate blood vessels. Data shown are representative images from n = 3 animals per group; experiments were repeated independently three times with similar results. One-tailed one-way ANOVA was performed followed by the Tukey test. Error bars, mean ± s.d. *P < 0.05, **P < 0.01, ***P < 0.001.
Significantly lower in the nonseeded group (0.4 ± 0.1 a.u.) of smooth muscle bundles, with less collagen content in the engineered uterus (0.05) than the nonseeded scaffold group (47 ± 1% versus 66 ± 6%, P < 0.05), and higher than the normal controls (47 ± 1% versus 25 ± 2%, P < 0.05) (Fig. 2m). The relative expression of αSMA by western blot analyses in the tissue-engineered group (8.8 ± 0.5 arbitrary units (a.u.)) and subtotal excision group (1 ± 0.2 a.u.) was similar to normal controls (1.2 ± 0.1 a.u., P = 0.16) and significantly lower in the nonseeded group (0.4 ± 0.2 a.u.; P < 0.01) (Supplementary Figs. 4a,b and 5 for full western blot gel image). The two-layered distribution of smooth muscle bundles in the neo-uterine tissue was confirmed using immunostaining for αSMA (Supplementary Fig. 4c).

We also evaluated the neo-uterine mucosa, including endometrium thickness, vascularization and gland density. Hematoxylin and eosin (H&E) cross-sections were used to measure endometrium thickness and gland density per field. CD31 immunostaining was used to assess blood vessel endothelium. Among experimental groups, only the tissue-engineered uterus group formed native-like epithelial crypts (Fig. 3a). In addition, the tissue-engineered uterus group had endometrial gland and blood vessel densities comparable to normal controls (P=0.98 and P=0.95, respectively) (Fig. 3b,j,k); and greater endometrium thickness than nonseeded scaffold group and subtotal excision-only controls (315.6 ± 129.5, 166.6 ± 48.1 and 167.1 ± 65.3 µm, respectively; P < 0.01) (Fig. 3i).

Table 1 | Reproductive outcomes in experimental rabbits

| Outcomes | Tissue-engineered uteri group (n = 14) | Noneedle scaffold group (n = 14) | Subtotal excision-only controls (n = 7) | Normal controls (n = 7) | P value |
|----------|--------------------------------------|----------------------------------|---------------------------------------|------------------------|---------|
| Uterine segment | Engineered | Native | Scaffold only | Native | Native | Native |
| Pregnancy rate | 4/14, 28.5%* | 6/14, 42.8%* | 0/14, 0% | 7/14, 50% | 1/7, 14.3% | 7/7, 100% | <0.01 |
| Term delivery | 3/4, 75% | 6/6, 100% | 0/14, 0% | 5/7, 71.4% | 0/1, 0% | 7/7, 100% | 0.093 |
| Average litter per pregnancy* | 1 | 1.4 ± 0.8 | N/A | 1 | 1 | 3.9 ± 1 | ≤0.001 |
| Offspring average body weight at birth (g) | 46.7 ± 9.5 | 42.6 ± 9 | N/A | 44 ± 11 | N/A | 45.5 ± 5.3 | 0.994 |

Engineered refers to the site receiving a cell-seeded bioengineered construct. Native refers to the site receiving the scaffold alone, without the cells. Scaffold only refers to the site receiving a cell-seeded bioengineered construct. Native corresponds to either the remaining normal uterine tissue proximal to the implantation site or the tissue in the normal controls. Scaffold only refers to the site receiving the scaffold alone, without the cells. *P<0.05. Normal controls versus tissue-engineered uteri group versus subtotal excision-only controls. °P<0.05. Normal controls versus non-seeded scaffolds group. ¥Fisher exact test. *Average litter per pregnancy: the average number of fetuses in a single-horn pregnant uterus. ¥P<0.05. Normal controls versus tissue-engineered uteri group. 𐄿Kruskal-Wallis test. °One-tailed one-way ANOVA test; critical level of significance, P<0.05. Data are presented as mean ± s.d.
Collectively, these results indicate that the tissue-engineered uteri group formed patent lumina with vascularized native uterine tissue-like structures. In contrast, the nonseeded scaffold group had a sparse cellular structure and lumen-occlusive scar formation. Histological features found in the subtotal excision-only controls showed a poorly formed uterine structure, consisting primarily of scar tissue.
Pregnancy outcomes. To investigate the in vivo functionality of the engineered uterine tissue, we conducted reproductive studies using fertile males for natural mating starting 6 months after uterine reconstruction procedures. Pregnancy studies were done in 42 of the 78 rabbits. Animals were assigned to four groups: the tissue-engineered uteri group (n = 14), nonseeded scaffold group (n = 14), subtotal uterine excision-only controls (n = 7) and normal controls (n = 7). The reproductive outcomes are depicted in Table 1. Pregnancies were identified 29–30 d after successful mating (identifying sperm in the vagina following mating) by visualizing fetal bone formation with standard X-rays. Location of the fetuses inside the uterus was investigated via computed tomography by identifying fetus location relative to the titanium clips-demarcated segment (Fig. 5a), which was then confirmed macroscopically during surgical delivery (Fig. 5b,c). Only the rabbits receiving the tissue-engineered constructs had normal pregnancies inside the reconstructed segment of the uterus (four out of ten rabbits). There were no congenital malformations found at necropsy (Fig. 5d), and the average delivered fetus body weights were comparable to normal controls (P = 0.994), suggesting that the engineered uter i supported normal fetal development. Viable pregnancies also occurred inside the remaining cervical end native uterine tissue, proximal to the reconstructed segments. The nonseeded scaffold constructs and subtotal excision controls had no fetal development or placenta tion at the reconstructed segment of the uterine horn. While placentation occurred around the circumference of the bioengineered construct segment, it was not possible to demarcate the exact line of distinction between the engineered tissue and native tissue (Fig. 5e). There was no macroscopic evidence of defects or herniation along the pregnant engineered uterus, and histological analyses confirmed normal uterine tissue structures (Fig. 5f,g) and placental development (Fig. 5h–k). The results indicate that the tissue-engineered uteri achieved reproductive function, and responded to the expansion and mechanical strains that occur during pregnancy, allowing appropriate growth and intruterine survival of the fetuses until late stages, supporting live births.

Discussion

We report a regenerative medicine-based approach to create functional neo-uterine tissues that support pregnancy to term and live births in a large animal model. Our results demonstrate that an autologous cell-seeded bioengineered uterine construct develops all uterine tissue layers, including a vascularized endometrium with secretary gland structures and two-layered myometrium, in 6 months after implantation in a subtotally excised rabbit uterine horn. We also show that intrinsic regenerative mechanisms alone do not support functional restoration of uterine tissue layers following a large excision. The fundamental role of the uterus is to allow embryo implantation, fetal nourishment, and growth. The advantages of using rabbits for reproductive studies include: (1) females are always in estrus and ovulation is induced by mating, resulting in an exactly defined pregnancy stage, (2) they have a short reproductive cycle (pregnancy lasts for 31 d) and (3) placent al morphology and function are similar to humans.

In the present study, 6 months postengraftment, only the tissue-engineered uter i group supported pregnancy and normal fetal development to term within the length of the reconstructed segment after natural mating, resulting in well-formed offspring with body weights similar to normal rabbits. Unlike rodents, leporine early embryo development differs and is more relevant, as the first contact with a receptive endometrium occurs in the antimesometrial side at the time of blastocyst attachment and a displacement over the mesometrial side subsequently occurs where the placenta eventually develops. All experimental animals had the antimesometrial side removed, but only the uter i with the construct seeded with cells, the bioengineered uteri group, achieved successful blastocyst-endometrium interactions that resulted in normal placentation in the mesometrial side and normal fetal development. Computed tomography imaging and macroscopic findings at delivery confirmed placentation along the mesometrial region of the tissue-engineered segment. Notably, it was not possible to demarcate the exact line between the bioengineered construct and the retained mesometrium, because the pregnant engineered tissue was indistinguishable from the native tissue. The bioengineered construct supported stretch-induced tissue expansion (more than ten times its weight) and remodeling that occurs during pregnancy to accommodate the growing fetus, placenta and amniotic fluid, and supported formation of viable offspring with body weights similar to those of the normal controls, which suggests normal placental function. Although the number of fetuses per pregnancy was lower than in the normal controls, the overall reproductive function of the tissue-engineered uteri was re-established, allowing fetal development and pregnancies to term with live births in four out of 14 cases. The lack of reproductive outcomes observed in the nonseeded scaffolds and subtotal excision-only uteri are likely due to the multiple luminal strictures and incomplete development of all the tissue structures, including endometrial glands, that were noted in the study.

Small uterine defects are able to regenerate intrinsically. The regenerative capacity of small uterine defects has been shown with various synthetic biomaterials, including biodegradable polyetherurethane/poly-L-lactide or nonbiodegradable polytetrafluoroethylene. Both scaffold grafts, without any added cells, led to endometrial regeneration in rodents. Naturally derived materials without cells have also been used as scaffolds for partial uterine reconstruction in rodents, including decellularized uterine tissue and boiled blood clot derived avascular scar tissue composed of myofibroblasts and collagen. Commercially available bovine derived freeze-dried collagen extract membranes, either with growth factors (bFGF), with cells derived from human embryonic stem cells, or with bone marrow derived stem cells, have been used for the replacement of uterine horn segments. Other studies using decellularized segments of uterine mucosa seeded with either rat uterine primary cells alone or together with commercially purchased rat MSCs also showed successful regeneration. In all the rodent studies described, when small uterine defects were created and repairs were attempted either with or without the use of naturally derived scaffolds, and either with or without the use of cells or growth factors, uterine regeneration with subsequent pregnancies were reported. Porcine small-intestinal submucosa grafts without the use of cells were also investigated for uterine reconstruction in rabbits. Similar to the rodent studies, the smaller grafts, smaller than or equal to 1 cm, supported reproductive function, and a common complication of a longer graft was graft collapse or twisting, confirming the inherent regenerative capacity of small uterine tissue segments and underscoring the challenges encountered when attempting to regenerate larger tissue defects. Tissue engineering strategies to replace critical-size defects and demonstrate clinically relevant regeneration are needed. In our studies in rabbits, synthetic biodegradable polymer scaffolds, 6–8 cm in length and 2.5 cm in width were used to engineer uterine tissue.

Synthetic polymer materials have been widely used as scaffolds for engineering tissues, are Food and Drug Administration-approved for human application, and are suitable for reconstructive surgery in selected patients. The advantage of using synthetic polymer scaffolds is that they can be produced on a large scale and controlled for physical properties such as tensile strength, degradation rate and three-dimensional design. We demonstrated that rabbit autologous primary uterine cells can be expanded ex vivo efficiently and successfully attach to PGA/PLGA scaffolds for large uterine reconstructive purposes. From an immunologic perspective, autologous cell sources are advantageous.
for the development of implantable-engineered tissues. The engineered uterine tissue supported pregnancy and normal fetal development to term, resulting in well-formed offspring. Our results introduce new avenues for potentially creating tissue substitutes derived from a patient’s own cells to treat uterine defects. Further preclinical studies are being planned before clinical trials are contemplated.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41587-020-0547-7.

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Methods

Animal protocol. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee and in accordance with all federal guidelines. Rabbits were euthanized according to the guidelines set forth by the American Veterinary Medicine Association.

Experimental design. Female New Zealand rabbits (3.5–4.5 kg) were purchased from Charles River Laboratories and single-housed at the Wake Forest University animal facility. They were fed standard rabbit chow (Purina), received water ad libitum, and were kept in rooms with automatically controlled temperature (22±0.1°C) and light-dark cycles (14 h of light and 10 h of dark). A total of 78 animals were randomly assigned to four groups: (1) a tissue-engineered uterine group (n=23) where the subbetaloplasty was repaired with an autologous cell-seeded scaffold; (2) a nonseeded scaffold group (n=23), where the subbetaloplasty was repaired with a polymer scaffold only; (3) a subtotal uterine excision-only control group (n=16), where the subbetaloplasty was repaired by suturing the remaining edges together and (4) a normal control group (n=16), where animals underwent a sham laparotomy.

Uterine tissue harvest and cell isolation. Under general anesthesia (2–5% isoflurane) and using aseptic technique, one uterine horn was identified through a midline laparotomy and completely excised from all rabbits. The uterine horn was transported in cold Dulbecco’s phosphate-buffered saline (DPBS) (14190136, ThermoFisher Scientific) supplemented with 1% antibiotic and antifungal (SV3007901, ThermoFisher Scientific). Using aseptic techniques, the uterine tissue was removed from the uterine dish, dissected free of any connective tissue, minced lengthwise, and washed with cold DPBS (14190136, ThermoFisher Scientific) to remove any debris. The endometrial lining was gently scraped off and minced into 1–2 mm³ pieces using a scalpel, and then transferred to a six-well tissue culture plate (353046, Corning). The myometrium tissue layer was then minced into 1–2 mm³ pieces and transferred to a different six-well culture plate (353046, Corning) for smooth muscle cell isolation. Three to four pieces of tissue were disposed per well to allow for optimal cell outgrowth.

After the explants adhered to the dish surface (5 min), endometrium culture media containing Dulbecco’s modified Eagle’s growth medium (DMEM) F12 (SH3026101, ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) (100–500, Gemini Bioproducts), epidermal growth factor recombinant human protein (5 ng ml⁻¹, PHG0315; ThermoFisher Scientific), bovine pituitary extract (40 ng ml⁻¹, 13020814, ThermoFisher Scientific) and 1% antibiotic/antifungal solution (SV3007901, ThermoFisher Scientific) was gently added to the endometrial samples in a separate culture dish, myometrium media containing high-glucose DMEM (SH302431S, ThermoFisher Scientific) supplemented with 10% FBS (100–500, Gemini Bioproducts) and 1% antibiotic/antimycotic solution (SV3007901, ThermoFisher Scientific) was added to myometrium tissue explants. The uterine explants were incubated in humidified chambers at 37°C and 5% CO₂ until cell outgrowth was observed (4–5 d), after which the residual tissue pieces were gently removed using sterile forceps. The initial stage of cell growth was termed passage 0. At days 3–7, 10, and 14, the cells were passed onward to a fresh culture; this process was repeated until complete saturation. The solvent was allowed to evaporate for 24–48 h before sterilization with ethylene oxide gas.

Uterine biopsy and immunohistological analyses. Under general anesthesia, animals were prepared for aseptic surgery and a lower midline incision was performed, submerging the semicircular scaffold in 5% PLGA solution until complete saturation. The scaffold was then removed from the uterine tissue and cultured for 4–5 d in humidified chambers at 37°C and 5% CO₂. A total of ten million cells per cm² of the uterine tissue were seeded into the scaffolds. The stepwise seeding process involved gently pipetting cells onto the scaffold surface in a uniform layer. On the outer side, cell seeding was carried out from the top convex surface outwardly to the concave surface; on the inner side, cells were seeded from the edges toward the concave surface. Seeded scaffolds were transferred to a 150-mm dish (BP140-02, Corning), placed inside a silicone frame (5827744, McMaster-Carr) to prevent contact to the bottom of the dish and incubated for 2 h at 37°C to allow for cell attachment before being immersed in culture media. The medium was changed every 24 h, and constructs were incubated in serum-free media for 24 h before implantation. Nonseeded scaffolds were prepared for implantation identical to that of the cell-seeded scaffolds except without the addition of cells.

Representative samples of the constructs were subjected to microstructural analyses; these samples were fixed in 2.5% glutaraldehyde, dehydrated in ethanol and dried overnight. The scaffolds were then sputter coated with gold (Hummer 23, Anatech) and imaged with a Hitachi S570 system SEM (Hitachi Hi-Teck), with an accelerated voltage of 25 kV and 5×120 magnification.

Uterine construct implantation. Under general anesthesia, animals were prepared for aseptic surgery and a lower midline incision was performed to expose the uterus. A semicircular full-thickness segment of 6–8 cm in length (depending on individual differences in the recipient uterus) was excised from the exposed uterine horn, retaining a native tissue strip of 2–3 mm in the mesometrial side (vascular pedicle) and 0.5–1 cm in length of the nearby native tissue proximally (cervical end) and distally (tubal end) for anastomosis (Fig. 1d–f). Uterine tissue harvest and engineered uterine construct implantation was performed as described above.

Blocking solution containing 0.1% Triton X-100 and 3% bovine serum albumin (A-9647, Sigma-Aldrich) for 1 h at room temperature. Uterine-derived cells were immunolabeled with the following primary antibodies: Rabbit polyclonal-conjugated goat IgG (1:100, CBL12610, Merck), vimentin (1:100, ab28028, Abcam), α-SMA (1:100, C2687, Sigma-Aldrich) for 1 h at room temperature; followed by incubation with fluorescein isothiocyanate-conjugated IgG (1:250, ab97092, Abcam) for 60 min. Negative controls included phycocyanin-labeled mouse IgG2b, K (Iotype 595929, BD Pharmingen) and fluorescein isothiocyanate mouse IgG2a (ab279092, Abcam). Samples were analyzed using a FACSCalibur instrument (Röntgen Dickinson Immunocytometry Systems).

Bioengineering autologous uterine tissues. Two mm-thick nonwoven biodegradable polymer membranes composed of PGA (Biomedical Structures) and PLGA (3.4:1) with a packing density of 60 mg cm⁻² were cut into 15 350 µm fibers, interfiber distance of 100–200 µm and 95% porosity were cut and coated with PLGA (50:50, Sigma-Aldrich) in chloroform (5% w/v). In a fume hood, the PGA membrane was completely immersed in 5% PLGA solution until completely saturated. The PGA-PLGA coated scaffold was allowed to evaporate for 30 min and then was configured into a semicircular shape, suturing the edges (6–0 Vicryl suture) using a Pasteur glass pipette (1367820B, ThermoFisher Scientific). A second coating process was performed, submerging the semicircular scaffold in 5% PLGA solution until complete saturation. The solvent was allowed to evaporate for 2 h, and the scaffolds were kept in a desiccator for 24–48 h before sterilization with ethylene oxide gas.

Polymer scaffold constructs were custom-made for each animal based on measurements taken at the excised uterine horn. The bioengineered constructs ranged from 5–12 cm in length and 2.5 cm in width. Before cell seeding, the sutures were removed using sterile techniques and scaffolds were pretreated with myometrium medium and incubated overnight in humidified chambers at 37°C and 5% CO₂. Third-passage myometrium-derived cells (8×10⁵ cells per ml) were seeded onto the outer side of the scaffolds and cultured for 4–5 d in humidified chambers at 37°C and 5% CO₂. The constructs were then turned over using sterile forceps and passage 3 endometrial-derived cells (8×10⁵ cells per ml) were seeded on the inner surface of the scaffold and incubated for additional 4–5 d in humidified chambers at 37°C and 5% CO₂. A total of ten million cells per cm² of the cell population were seeded into the scaffolds. The stepwise seeding process involved gently pipetting cells onto the scaffold surface in a uniform layer. On the outer side, cell seeding was carried out from the top convex surface outwardly along the length of the scaffold; and in the inner surface, cells were seeded from the edges toward the concave surface. Seeded scaffolds were transferred to a 150-mm dish (BP140-02, Corning), placed inside a silicone frame (5827744, McMaster-Carr) to prevent contact to the bottom of the dish and incubated for 2 h at 37°C to allow for cell attachment before being immersed in culture media. The medium was changed every 24 h, and constructs were incubated in serum-free media for 24 h before implantation. Nonseeded scaffolds were prepared for implantation identical to that of the cell-seeded scaffolds except without the addition of cells.

Representative samples of the constructs were subjected to microstructural analyses; these samples were fixed in 2.5% glutaraldehyde, dehydrated in ethanol and dried overnight. The scaffolds were then sputter coated with gold (Hummer 6.2, Anatech) and imaged with a Hitachi S570 system SEM (Hitachi Hi-Teck), with an accelerated voltage of 25 kV and 5×120 magnification.

Uterine morphology and immunohistological analyses. Thirty-six of the 78 animals were killed at 1, 3 or 6 months after scaffold implantation surgery for morphological and histological analyses (n=3 per experimental group per time point).
point) after scaffold implantation surgery. Before euthanasia, hysterograms were performed under fluoroscopy to assess uterine lumen characteristics. Animals were anesthetized, placed on a fluoroscopy table, and diluted (1:10) contrast medium (Conray, 50% iohexol methylglucamine injection USP 60%) was instilled into the uterine cavity through the cervical canal via a cannula. Digital fluoroscopic images were acquired using the Siremobil compact L system (Siemens).

Tissue samples from the engrafted area were retrieved for histological assessment. Specimens were fixed in 10% neutral buffered formalin for 48 h, processed (ASP3905S; Leica Biosystems), and paraffin-embedded (EG1160; Leica Biosystems). Serial paraffin cross-sections (7 μm) were prepared and either stained with H&E using an automated stainer (ST9010 autostainer XL, Leica Biosystems) or Masson’s Trichrome. Three slides containing cross-sections from the retrieved tissues (n = 3 per experimental group per time point) were imaged using a motorized inverted microscope (Olympus IX83), and digitized for further morphometric analysis. The endometrial thickness was estimated by measuring the points of greatest perpendicular depth (n = 4 per field) at the antimesometrial area from the luminal surface to the endometrium–myometrium interface under a magnification of x100. To determine uterine gland abundance, microscopic fields were randomly selected in the lateral and antimesometrial sides of each section using a ×20 objective, and the number of uterine gland cross-sections was calculated and normalized per endometrial area.

Images captured from Masson’s Trichrome-stained slides (under a magnification of x100) were used to determine the relative content of collagen/ connective tissue (blue) and smooth muscle (red) in the uterine tissue. The relative collagen content was calculated as the total amount of blue stained areas divided by the sum of all red and blue stained areas in the regions of interest in three high-power fields. The uterine lumen and blank areas were subtracted from all calculations for accuracy.

For immunohistochemistry, sections were blocked with avidin/biotin kit (SP-2001, Vector Laboratories) and incubated overnight at 4 °C with anti-α-SMA (1:40, ab81847, Abcam), anti-uteroglobin (7G4E9, 1:100, ab50711, Abcam), anti-CD31/PECAM-1 (C317, NB2-15188, Novus Biological), antiestrogen receptor alpha (sc-5002, Santa Cruz) and anti-progesterone receptor (sc-811, Santa Cruz) separately. Next, biotinylated goat anti mouse IgG (BA 9200, 1:300, Vector Laboratories), biotin-streptavidin HRP complex and DAB chromogen (SK-415, Vector Laboratories) were incubated with peroxide-conjugated goat-anti rabbit IgG (ab-7187, Abcam) were diluted in blocking solution and incubated with membranes for 1 h at room temperature. Membranes were blocked with 5% milk powder for 1 h at room temperature. Primary antibodies anti β-actin (1:200, sc-47778, Abcam) and anti-α-SMA (1:200, ab-7187, Abcam) were diluted in blocking solution and incubated with membranes for 1 h at room temperature. Membranes were incubated with peroxide-conjugated bovine anti goat secondary antibody (1:1000, sc-2378, Santa Cruz Biotechnology) and stained with enhanced chemiluminescent substrate (Pierce) and protein signals were visualized with a LAS-3000 imaging system (Fujiﬁlm). Images were densitometrically scanned using Image J (National Institutes of Health) and protein quantification was analyzed with GraphPad Prism 8.0 for Windows (GraphPad Software). All densitometric raw data values for α-SMA were normalized to β-actin from the same sample lanes. Thus, a ratio of the amount of α-SMA, relative to an internal calibration of β-actin, was calculated for each group.

In vivo reproductive study. Rabbits were naturally mated with fertile New Zealand male rabbits 6 months after undergoing the scaffold implantation procedure. Mating occurred in a breeding cage and was confirmed by observing male mounting behavior and the presence of sperm in vaginal smears. The mating day was considered gestation day zero, and the number of fetuses was confirmed on standard radiographs taken at days 29–30. Pregnant rabbits were scanned using a computed tomography system (Aquilion 32, Toshiba) with a three-dimensional body image reconstruction system to determine the fetus’ positions in relation to the titanium clips placed around the bioengineered cell-seeded constructs.

Rabbit delivery/Cesarean section. On days 29–30 of gestation, rabbits were administered buprenorphine (0.03 mg kg⁻¹ intramuscular) for sedation and general anesthesia was induced and maintained on a ventilator with inhaled isoflurane (2–5%) and oxygen. Following a midline laparotomy, the pregnant uterus was exposed and examined for number of fetuses in relation to the engrafted site and uterine location. The viability and body weights of the delivered offspring were assessed at birth. All animals were euthanized following surgical delivery, and gross morphopathological analysis of the offspring was conducted by an independent pathologist.

Statistical analysis. All values are expressed as the mean ± s.d. For comparison of continuous variables across multiple groups, one-way analysis of variance (ANOVA) was performed followed by the Tukey test. Nonparametric analogs were applied for ordinal categorical variables. Fisher’s exact test was performed to compare pregnancy rates. Statistical analyses were conducted using the R computer software for Windows (v.3.2.3). A P value less than 0.05 was considered statistically significant. Due to the exploratory nature of the study, no adjustment for multiplicity was applied.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that supports the findings of this study are available from the corresponding author, upon reasonable request.

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Author contributions. A.A. developed the concept of engineering functional autologous uterine tissue constructs. R.S.M., J.W., J.J.Y. and A.A. designed all experiments. R.S.M. performed the in vitro experiments and fabricated the bioengineered uterine constructs. R.S.M. and J.K.W. performed in vivo experiments of uterine tissue excision and construct implantation. K.W.Y. performed in vitro experiments and analyzed the data. R.S.M., J.K.W. and A.A. analyzed the data and wrote the manuscript. A.A. provided direction and supervised the project. R.S.M., J.K.W., J.J.Y. and A.A. reviewed and edited the manuscript.

Competing interests. Boston Children’s Hospital was assigned the rights to two issued patents, both titled “Tissue Engineered Uterus,” no. 7,049,057, filed 15 November 2002, and no. 7,429,490, filed 7 February 2005, with A.A. and J.J.Y. listed as inventors. Based on the remaining patent term and the need for further studies before this technology is used clinically, there are no current or expected financial interests related to these patents.

Additional information. Supplementary information is available for this paper at https://doi.org/10.1038/s41587-020-0547-7. Correspondence and requests for materials should be addressed to A.A. Reprints and permissions information is available at www.nature.com/reprints.
Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

- Data collection: Reported on page 30 (Methods Section)
- Data analysis: R computer software for Windows (version 3.3.2) was used for statistical analysis

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
The number of animals was derived from a Power calculation based on our preliminary data for differences in means, standard deviations, and assuming power=0.8 and alpha =0.05

**Data exclusions**
Animals with urogenital, reproductive, or clinical problems were not included in the study

**Replication**
Technical replicates were performed 3 times

**Randomization**
Animals were randomly assigned to experimental groups upon arrival to the animal facility

**Blinding**
Investigators were blinded during histological and morphometrical data analysis

Reporting for specific materials, systems and methods

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### Materials & experimental systems
- n/a Involved in the study
  - X Antibodies
  - □ Eukaryotic cell lines
  - □ Palaeontology
  - □ Animals and other organisms
  - □ Human research participants
  - □ Clinical data

### Methods
- n/a Involved in the study
  - X ChIP-seq
  - □ Flow cytometry
  - □ MRI-based neuroimaging

**Antibodies**

| Antibodies used | Validation |
|-----------------|------------|
| Anti-pan cytokeratin (AE1/AE3, 1:50, ab28028; Abeam), vimentin (1:100, ab28028; Abeam), smooth muscle myosin heavy chain 11 (MHC, 1:250 ab6883; Abeam), calponin (1:100, C2687; Sigma-Aldrich), anti-a-SMA (1:40, ab18147; Abeam), anti-uteroglobin (7G4E9, 1:100, ab50711; Abeam), anti-CD31/PECAM-1 (C31.7, NBP2-15188; Novus biological), anti-estrogen receptor alpha (sc-5002; Santa Cruz), and anti-progesterone receptor (sc-811; Santa Cruz), biotinylated goat anti-mouse IgG (BA 9200, 1:300; Vector laboratories), goat anti-mouse Alexa Fluor 488 (1:500, All0l 7; ThermoFisher), Alexa Fluor 594 (1:100, A11020; ThermoFisher), R-phycoerythrin-conjugated CD9 (1:100, CBL162P; Millipore), fluorescein isothiocyanate (FITC)-conjugated IgG (1:250, ab79092; Abeam), phcoerythrin-labeled mouse IgG2a, K Isotype (559529; BD Pharmingen), and FITC mouse IgG2a (ab79092; Abeam), anti-?-actin (1:200, sc-47778; Abeam), Anti-a-SMA (1:200, ab-7187; Abeam), and bovine anti-goat secondary antibody (1:1000, sc-2378; Santa Cruz Biotechnology) |
| Primary antibodies were validated in normal rabbit uterine tissue ( anti-pan cytokeratin (AE1/AE3), vimentin, smooth muscle myosin heavy chain 11, calponin, anti-a-SMA, anti-estrogen receptor alpha, and anti-progesterone receptor, and CD9 ), rabbit aorta (anti-CD31/PECAM-1 ) and rabbit lung tissue (anti-uteroglobin) , respectively according to the manufactures' website. |

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Wild animals |
|--------------------|--------------|
| Adult female New Zealand white rabbits (3.5-4 kg) | The study did not involve wild animals |

| Field-collected samples | Ethics oversight |
|-------------------------|-----------------|
| The study did not involve samples collected from the field | All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee and in accordance with all federal guidelines. Rabbits were euthanized according to the guidelines set forth by the American Veterinary Medicine Association. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
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- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

| Sample preparation       | Reported on pages 22-23 (Methods Section) |
|--------------------------|-------------------------------------------|
| Instrument               | FACSCalibur instrument (Becton Dickinson Immunocytometry Systems) |
| Software                 | BD Cell Quest Pro software (version 5.1) |
| Cell population abundance| Cell were not sorted in this study          |
| Gating strategy          | Cell populations were gated out on the basis of scatter properties, excluding debris and doublets |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.