**Title:** A rat model of male pregnancy

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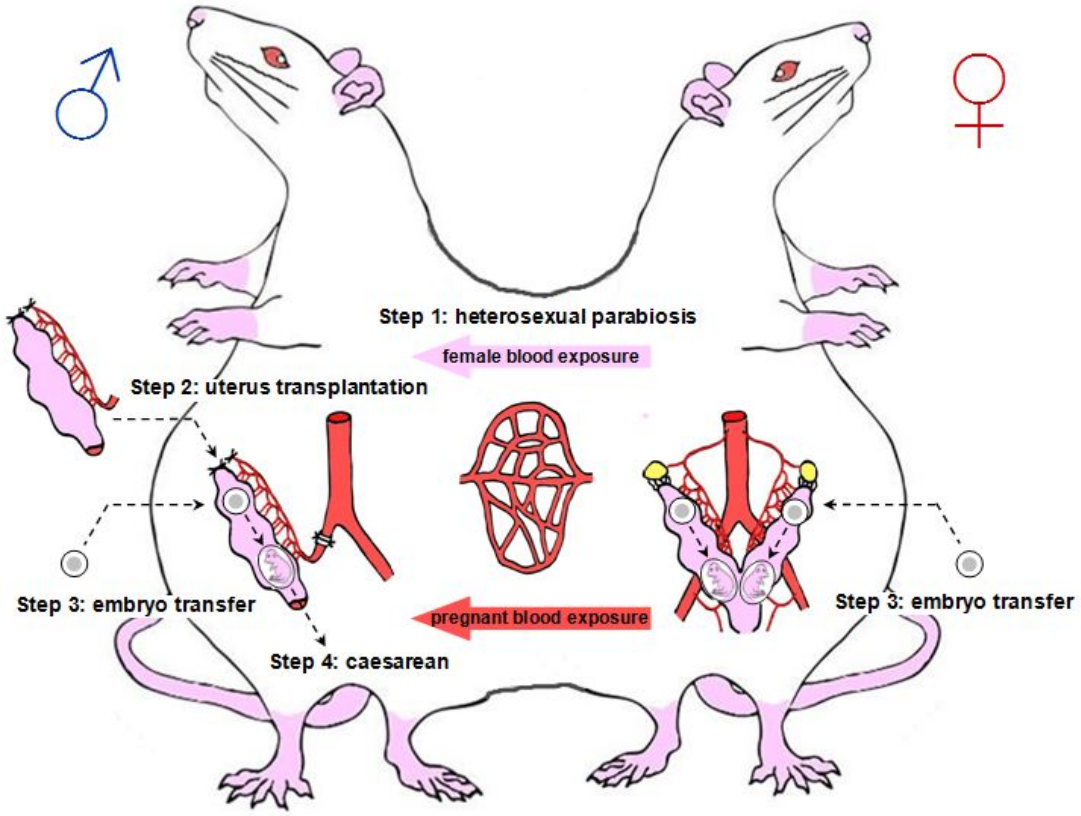
**Abstract:** Male pregnancy is a unique phenomenon in syngnathidae which refers to the incubation of embryos or fetuses by males. However, whether male mammalian animals have the potential to conceive and maintain pregnancy remains unclear. Here, we constructed a rat model of male pregnancy by a four-step strategy: a heterosexual parabiotic pair was firstly produced by surgically joining a castrated male rat and a female rat. Uterus transplantation (UTx) was then performed on the male parabiont 8 weeks later. After recovery, blastocyst-stage embryos were transplanted to the grafted uterus of male parabiont and the native uterus of female parabiont. Caesarean section was performed at embryonic day (ED) 21.5. The success rate of modeling was only 3.68%, but 10 pups could still be delivered from male parabionts and developed. Our experiment reveals the possibility of normal embryonic development in male mammalian animals, and it may have a profound impact on reproductive biology.

**One-sentence summary:** A rat model of male pregnancy can be constructed in four steps.

**Graphical abstract:**



## MAIN TEXT

### Introduction

Male pregnancy is an extremely rare phenomenon in nature which generally refers to the incubation of embryos or fetuses by males until birth (*1*). Syngnathidae is the only known family of maturing their offspring by males during pregnancy (*2, 3*). In mammalian animals, pregnancies are carried out by females. However, whether male mammalian animals have the potential to conceive and maintain pregnancy remains unclear. It was reported that mouse embryos transferred to non-uterine organs of male hosts will only develop into a limited stage (*4, 5*), suggesting some factors may restrict a complete development of embryos in male mammalian bodies. We speculate two points may be responsible: 1, male mammalian animals have no uterus for embryo implantation and development; 2, male mammalian animals lack the specific female and pregnant microenvironment (dynamic levels of hormone and other molecules in the body) which promote endometrial growth and allow embryo implantation or development.

Here we investigated the question of whether male pregnancy with livebirths can be achieved in a rat model if the speculative problems were solved by existing methods. Accordingly, uterus transplantation (UTx) and heterosexual parabiosis were incorporated into our experimental protocol. UTx is a surgical procedure which has been conducted in several species of mammalian animals (*6-12*). Parabiosis is an experimental model which can surgically connect two animals and share their blood microenvironment through anastomosis (*13*). In our experiment, a four-step strategy was planned: 1, a male rat was subjected to receive castration and joined with a female rat (named heterosexual parabiosis) (*14*) to obtain the similarly female microenvironment by blood exchange; 2, UTx was then performed on the male parabiont; 3, for observing embryonic development in the grafted uterus of male parabiont under pregnant blood exposure, blastocyst-stage embryos were transferred to both the grafted uterus of male parabiont and the native uterus of female parabiont; 4, caesarean section was performed if the male parabiont was pregnant.

### Results

#### Modeling and screening of heterosexual parabiotic pairs

The first step of our experiment is modeling and screening of heterosexual parabiotic pairs (Fig. 1A). To reduce the possible immune rejection caused by parabiosis and subsequent UTx, we chose inbred Lewis rats as the object. Firstly, all the female and male Lewis rats were screened preliminarily. By two weeks of vaginal smear observation, the female rats with three regular estrous cycles which each cycle lasted four days and was presented as E-M-D-P or E-M-M-P or E-D-D-P were included in our experiment (Fig. 1B and 1C). Those female rats with no obvious estrous cycles or with irregular estrous cycles were excluded (Fig. 1C). Then the selected female rat was divided into two parts: 1, as donor for UTx; 2, as female parabiont for heterosexual parabiosis surgery. Meanwhile, by mating with superovulated female rats, male rats with verified reproductive function were proposed as the male parabiont. Before parabiosis surgeries, the testes, epididymes, right ventral prostate and seminal vesicles were removed (Fig. 1D). Two weeks after parabiosis surgeries (Fig. 1D and S1A), both female donors and female parabionts were received estrous cycle synchronizations, and only those with three synchronized estrous cycles examined by vaginal cytology could proceed to the next step.

To explore whether male parabionts were under female blood exposure after parabiosis surgeries, we examined serum levels of progesterone and estrogen-17β in both male and female parabionts. Late stage of metestrus and late stage of proestrus were chosen as the time points (Fig. S1B), because they are the peaks of hormone curve in female rats (*15*). For female rats exhibited all three E-M-D-P estrous cycles before parabiosis surgeries, levels of both progesterone and estrogen-17β were significant different between in late metestrus and in late proestrus. But the changes were not observed in male rats at the same time points (Fig. 1E). Six weeks after parabiosis surgeries, while female parabionts maintained the significant hormone alternations, male parabionts also exhibited the similar significant trend (Fig. 1E). To verify that the acquired hormone alternations in male parabionts are induced by female blood exposure from female parabionts, separation surgeries were performed at eight weeks after parabiosis

surgeries (Fig. 1D). We found that levels of progesterone and estrogen-17β in female rats still kept the significant changes between in late metestrus and in late proestrus at two weeks after the separation, but the male rats lost the significant alterations (Fig. 1E). Additionally, to investigate whether female parabionts were affected by androgen from male parabionts after parabiosis surgeries, serum levels of testosterone were also detected. We found that the testosterone levels was significantly decreased by castration in male parabiont at eight weeks after parabiosis surgeries, and no significant alternations were observed in female parabionts (Fig. S1C).

**Grafted uteruses transplanted into male parabionts**

The second step of our experiment is to transplant grafted uteruses into male parabionts of heterosexual parabiotic pairs (Fig. 2A). We devised a novel UTx protocol which anastomoses the right common iliac vessels of grafted uteruses with the right common lilac vessels of male parabionts by end-to-end cuff technology (Fig. 2B) (*16-18*). However, a possibility should be considered that the surgical program may lead to right hindlimb ischemia and ultimately impair the function of grafted uterus in male parabionts. Thus, we firstly explored the effect of ligation and cutting of right common iliac artery and vein on the right hindlimb in male rats, and found that there was no obvious ischemia or necrosis on the right hindlimb at 8 weeks after the surgery (Fig. S2A). Next we ligated and cut off the right iliac vessels of female rats but kept the branch supplying the uterus, and also found no visible hindlimb ischemia or necrosis at 8 weeks after the surgery (Fig. S2B). Then those female rats underwent ligation and cutting were mated with normal male rats. We found that ligation and cutting of right iliac vessels but kept the branch supplying the uterus did not significantly impair female fertility (Fig. S2C) and change weight evolution of pups (Fig. S2D). Thus, we can infer that our UTx protocol may not cause hindlimb ischemia and uterine damage at 8 weeks after UTx.

Next we implemented our UTx protocol by cuff technology (Fig. S3). The uterine graft was firstly isolated in the female recipient, and then cuff preparation, cuff anastomosis, cuff reperfusion, uterine localization and ostomy were performed in the male parabiont (Fig. 2C). However, before the formal surgery of UTx started, we had conducted a certain degree of surgical training to improve the success rate (Fig. S4). To reduce the number of animals used in surgical training, male individuals rather than male parabionts of heterosexual parabiotic pairs were chosen as the recipients (Fig. S4A). We divided the surgical training into two stages, and the formal UTx was performed after ensuring that the time of warm ischemia-reperfusion (I/R) can be controlled within 30 min (Fig. S4B-S4E). After the formal UTx, the skin around the stoma was sutured with gauzes and a long-term care was performed (Fig. S5A and S5B). During the recovery, estrous cycles of female parabionts were monitored, and the female parabiont was received hormonal regulation if the estrous cycle was unusual (Fig. 2A). Eight weeks after UTx, surviving heterosexual parabiotic pairs with normal estrous cycles in female parabionts could proceed to the next step.

The functional recovery of grafted uterus is closely related to immune rejection (*19, 20*) and I/R injury (*21*). Thus, H&E and $CD8^+$ immunohistochemical staining were performed at 8 weeks after UTx. We found that no large area of necrosis (typical features of immune rejection) (*19, 20*) and obvious extravasation of blood and severe loss of endometrium (typical features of I/R) (*21*) were observed in grafted uteruses of male parabionts (Fig. 2D). No significant differences of $CD8^+$ count can be found in grafted uteruses of male parabionts compared with native uteruses of female individuals and female parabionts, respectively (Fig. S5C and S5D). To further investigate whether grafted uteruses of male parabionts were influenced by female blood exposure from female parabionts, we examined both grafted and native uteruses by electron microscopy at different estrous stages of female parabionts. In accordance to previous electron microscope results (*15*), the native uterus of female parabiont has the characteristic ultrastructures according to different estrous stages, and the grafted uterus of male parabiont also presented the similar phenomenon expectedly (Fig. 2E and S5E).

**Transferred embryos developed in grafted uteruses of male parabionts under pregnant blood exposure from female parabionts**

The third step of our experiment is to transplant blastocyst-stage embryos to both grafted uteruses of male parabionts and native uteruses of female parabionts (Fig. 3A).

Three days before embryo transfer, female parabionts were mated with vasectomized male rats to achieve pseudo-pregnant female blood exposure to male parabionts. To increase the success rate of mating, the vasectomized male rats proposing for mating had been trained and screened before vasectomy (Fig. 3B). On the day of embryo transfer, blastocyst-stage embryos were firstly collected from treated female rats, and then the heterosexual parabiotic pairs were subjected to laparotomy to check whether the morphology and color of grafted uteruses of male parabionts were similar to native uteruses of female parabionts (Fig. 3C). After that, embryos were transplanted to left uteruses of female parabionts, grafted uteruses of male parabionts, and right uteruses of female parabionts, respectively (Fig. S6A). Immunosuppression and stoma care were still performed after embryo transfer (Fig. S5B).

A total of 842 blastocyst-stage embryos had been transferred to 46 heterosexual parabiotic pairs at embryonic day (ED) 4.5 (562 embryos transferred to female parabionts and 280 embryos transferred to male parabionts). At ED 18.5, exploratory laparotomy was performed to observe the development of transferred embryos (Fig. 3C). We found that 169 (30.07%) embryos developed normally in native uteruses of female parabionts at ED 18.5, while only 27 (9.64%) embryos developed normally in grafted uteruses of male parabionts (Fig. S6B). Further mining the data indicated that all those developing embryos in male parabionts had been exposed to pregnant blood environment from female parabionts (Fig. S6C). In heterosexual parabiotic pairs, 25 (54.35%) pairs showed no normal embryos in both male and female parabionts (Fig. 3C and movie 1); 15 (32.61%) pairs exhibited at least one normal embryo only in female parabionts (Fig. 3C and movie 2); 6 (13.06%) pairs presented at least one normal embryo in both male and female parabionts (Fig. 3C and movie 3); no (0%) pair displayed at least one normal embryo only in male parabionts (Fig. 3D). We thus inferred that the transplanted embryos may develop normally in grafted uteruses of male parabionts only when the female parabionts conceive and provide pregnant blood exposure to male parabionts. To verify our speculation, 90 blastocyst-stage embryos were only transplanted to grafted uteruses of male parabionts at ED 4.5 (n=15). Consistent with our speculation, no normal developing embryos were found in grafted uteruses of male parabionts at ED 18.5 (Fig. 3D and S6C).

**Surviving fetuses and male parabionts after caesarean sections**

The last step of our experiment is observing the pregnant male parabionts and their surviving fetuses after caesarean sections (Fig. 4A). We firstly performed caesarean sections on pregnant female individuals copulated normally at ED 21.5, and found that all fetuses survived at the time of caesarean section, but some of them died 2 hours later (Fig. 4B). The cause of death might be the early termination of pregnancy induced by caesarean sections. Next we performed caesarean sections on those heterosexual parabiotic pairs which both male and female parabionts were pregnant. Except for a resorbed fetus, all fetuses born from female parabionts were alive after caesarean sections, but some of them still died 2 hours later (Fig. 4B and 4C). However, during the caesarean sections of male parabionts, we found some abnormal dead fetuses that had never occurred in other two groups (Fig. 4B). The typical characteristics of these dead fetuses are: 1, different morphology and color compared with normal fetuses; 2, placentas atrophy or swelling. Surviving fetuses and a small number of resorbed fetuses could also be delivered by caesarean sections from male parabionts, and some fetuses were still alive 2 hours later (movie 4). The body weight and placental weight of live fetuses in male parabionts were not significant different compared with other two groups at 2 hours after caesarean sections (Fig. 4C). These newborn pups born from male parabionts could also develop normally to maturity with the reproductive function (Fig. S7A, S7B and 4E), and histological examinations showed that their heart, lung, liver, kidney, brain, testis, epididymis, ovary and uterus had no obvious abnormalities (Fig. S7C and 4D).

After caesarean sections, we performed separation surgeries on the heterosexual parabiotic pairs, and found that all separated male parabionts could survive 3 moths after the surgeries. Then we used karyotype analysis on these separated male parabionts to determine their chromosomal sex. We firstly chose normal male individuals (no treatment after the castration in Step 1) as the references. Although the karyotype results may change in inbred rats (*22*), no obvious difference could be found in sex chromosomes between the separated male parabionts and normal male individuals (Fig. 4F), suggesting

that the chromosomal sex of these separated male parabionts was indeed male.

## Discussion

To our best knowledge, it has never been reported before that male pregnancy can be achieved in mammalian animals. Here we constructed a rat model of male pregnancy, and found that the transplanted blastocyst-stage embryos may develop to maturity in grafted uteruses of male parabionts if the male parabionts are under pregnant blood exposure form female parabionts. The success rate of the entire experiment was very low, but 10 pups could still be delivered from male parabionts by caesarean sections and developed into adulthood (Fig. S8). Additionally, we found two new phenomena in our rat model of male pregnancy. First, during caesarean sections at ED 21.5, abnormal dead fetuses were only observed in grafted uteruses of male parabionts. Considering no abnormalities could be found during the laparotomy at ED 18.5, it was inferred that the abnormal death of fetuses in male parabionts began in the late stage of embryonic development (approximately at ED 18.5-21.5). Whether this phenomenon is peculiar to male pregnancy in mammalian animals remains unknown. Second, only those embryos exposed to pregnant blood from female parabionts may develop normally in male parabionts, suggesting the normal development of embryos in male mammalian animals rely on a mechanism that can be induced by pregnant blood exposure rather than female blood exposure. The specific mechanism still needs further investigations.

For the first time, a mammalian animal model of male pregnancy was constructed by us. Our research reveals the possibility of normal embryonic development in male mammalian animals, and it may have a profound impact on the research of reproductive biology.

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## Materials and methods

### Animal housing and parabiosis care

All male and female inbred Lewis rats were obtained from Shanghai Sippr-BK laboratory animal Co. Ltd. and housed in animal experimental center of Naval Medical University and experimental teaching center of Facility of Naval Medicine. They were maintained in a 12-h light-dark cycle with free access to food and water. Parabiotic pairs were housed individually to avoid animal biting. To reduce wound infection induced by

faecalis adherence, the padding was replaced once a day within the first 3 days after the surgery and was changed once every 3 days during the remaining time. All experimental procedures were performed according to local ethics guidelines on animal welfare and permits.

**Vaginal cytology for estrous cycle determination**

Female rats were monitored for estrous cycles for 14 days using vaginal cytology. Vaginal cytology was examined once daily at approximately 8:00 a.m. by collecting vaginal secretions using sterile plastic pipettes filled with saline. Vaginal fluid was placed onto glass slides and fixed by adding a drop of 95% alcohol. Then the slides were stained by Hematoxylin-Eosin and examined under a microscope. The subjects were recorded as being in diestrus, proestrus, estrus, metestrus based on the proportion of cell types. A diestrus smear primarily consists of a predominance of leukocytes; a proestrus smear consists of a predominance of nucleated epithelial cells; an estrous smear primarily consists of a nucleated cornified cells; a metestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells (*23*).

**Heterosexual parabiosis and division surgery**

For heterosexual parabiosis surgery, both male and female rats were anesthetized by isoflurane (5% for induction, 2% for maintenance, 2 l/min of oxygen) (*24*) and placed in a supine position. A laparotomy was performed to the male rat through a mid-line incision. Bilateral testes and epididymes were removed from male rats to reduce the effect of androgens on female parabionts. A resection of seminal vesicles was performed to avoid the false positive result from occurred vaginal plug during mating of female parabionts and sterilized male rats in Step 3 (Fig. 3A). Right ventral prostate was also removed to avoid physical contact with grafted uteruses. Then skin incisions were made along the lateral surface of both male and female rats. Parabiotic pair was generated by first suturing the opposing flanks of elbows and knees and then connecting the dorsal and ventral skin (Fig. S1A). After the surgery, both male and female parabionts were received a dose of ampicillin (20 mg/kg) to prevent infection. Isosexual parabiotic pair (female-female) was performed as the similar way. For separation surgery, heterosexual parabiotic pairs were also under isoflurane anesthesia (5% for induction, 1.5% for maintenance, 2 l/min of oxygen). Then all remaining sutures were removed, and the connected skin and joints were separated carefully. Animal's body temperature was controlled by a thermostatically heated pad.

**Blood collection for ELISA**

Female blood samples were collected in late metestrus and in late proestrus from female rats or female parabionts that had all three E-M-D-P cycles. Male blood samples were collected at the same time. To speculate the approximate time of late metestrus and late proestrus, we drew a figure of female estrous cycles according to 14 days of vaginal smears (Fig. S1B). Vaginal cytology was examined at 8:00 a.m. and 4:00 p.m. on the speculated day and at 8:00 a.m. on the next day. If the females were in metestrus or in proestrus at both 8:00 a.m. and 4:00 p.m. on the speculated day, blood samples were then collected from the orbital venous plexus at approximately 4:30 p.m.. If the females were in diestrus or in estrus at 8:00 a.m. on the next day, the samples collected yesterday were finally included. The levels of progesterone, estrogen-17β and testosterone in blood were measured using ELISA kits (progesterone, Biovision, K7416-100; estrogen-17β, Sigma, ab108667; testosterone, Cayman, 582701) according to the manufacturer's instructions. Absorbance was measured at 405 nm with the correction set at 600 nm.

**Uterus transplantation (UTx)**

*Donor preparation and operation*

The Female donor was received luteinizing hormone releasing hormone agonist (LHRHa) (*25*) for estrus synchronization (Fig. 1A). UTx was performed when the female donor was in diestrus. The donor was anesthetized by isoflurane and then a midline laparotomy was performed. The right uterine horn with the right uterine cavity plus a vascular pedicle including the right uterine and common iliac vessels was separated (Fig. 2C1) according to the schematic drawing (Fig. 2B). After ensuring no bleeding points of separated tissues, a piece of sterile gauze was soaked in 37°C saline and covered the incision.

*Recipient preparation and operation*

The Female parabiont was received LHRHa for estrus synchronization (Fig. 1A). UTx was performed on the male parabiont when the female parabiont was in diestrus. The parabiotic pair was anesthetized by isoflurane and then a midline incision was made to the male parabiont. After the right common iliac artery was exposed, the proximal portion was clipped with a microvascular clamp, the distal portion was ligated with a 4-0 silk thread, and the right common iliac artery was cut off between the ligature and the clip. The proximal end was flushed with sodium heparin saline (200 IU/mL), and the right common iliac artery was passed through a well-designed cuff (Fig. S3A and Fig. S3B). The artery was then folded over the cuff and secured with a 8-0 nylon tie to expose the endothelial surface under a dissecting microscope (Hotry, MT-1). The right common iliac vein of male parabiont was treated in the similar way (Fig. 2C2).

*Vascular anastomosis, vaginal ostomy, immunosuppression and stoma care*

In female donors, the right common iliac artery and vein were cut respectively. The uterine graft was harvested and flushed by ice-cold heparin sodium saline (200 IU/mL) containing dissolved hydrogen (1.8 mg/L, to reduce I/R injury) (*26*). By using a dissecting microscope, the graft's right common iliac artery was then placed over the male parabiont's arterial cuff and fixed with a 5-0 silk thread. The similar procedure was performed to fix the right common iliac vein on the venous cuff (Fig. 2C3 and S3C). After unclamping, the uterine grafted was restored blood supply (Fig. 2C4). The adipose tissue near uterine horn was then fixed on the connective tissue under the right kidney (Fig. 2C5), and the vaginal end was everted to form a stoma (Fig. 2C6). After closing the midline incision, the skin around the stoma was sutured and fixed with a sterile gauze and a zinc oxide gauze (Fig. S5A), and both male and female parabionts were received a dose of ampicillin (20 mg/kg) and tacrolimus (0.2 mg/kg, Abcam, ab120223) (*20*). Stoma care (dressing change) and immunosuppression (0.1 mg/kg tacrolimus) should be kept during the remainder of the experiment according to our protocol (Fig. S5B).

**Hindlimb ischemia**

Experiments were performed on adult male rats and virgin female rats exhibited normal estrous cycles. For male rats, the right common iliac artery and venous were ligated below the bifurcation from the abdominal vessels under isoflurane anaesthesia. Observations were performed at 8 weeks after surgeries. For female rats, the right common iliac artery and venous were ligated below the uterine artery and venous under isoflurane anaesthesia. Observations were also performed at 8 weeks after surgeries, and then superovulations were carried out. For mating, each superovulated female rat has two chances to mate with a male rat experienced reproductive success.

**Histology and transmission electron microscopy**

Animals were perfused transcardially with saline followed 4% paraformaldehyde under anaesthesia. The uterus was then removed and fixed in 4% paraformaldehyde. For histological evaluation, the tissue was dehydrated, infiltrated, embedded, sectioned (4 μm), and stained with hematoxylin-eosin (H&E) or CD8 immunohistochemistry (Abcam, ab237709). Sections were examined using a computer image analysis system (Kongfoong, KF-PRO-120) in a blinded fashion. Transmission electron microscopy was performed on those heterosexual parabiotic pairs that female parabionts had all three E-M-D-P cycles after vaginal cytology. Briefly, the tissue was post-fixed in 1% osmiumtetroxide, dehydrated, infiltrated, embedded, trimmed, sectioned (70 nm), and stained with acetate double oxygenic uranium and lead citrate. Image acquisition and analysis were performed in a blinded way using a transmission electron microscope (Hitachi, H7650).

**Embryo transfer**

*Collection of blastocyst-cell stage embryos*

Adult female Lewis rats were stimulated for superovulation by an intraperitoneal (IP) injection of 15 IU pregnant mare's serum gonadotropin (PMSG, BioVendor R&D, RP17827210000) followed 48 hours later by an IP injection of 20 IU human chorionic gonadotropin (hCG, Sigma, 9002-61-3) (*27*). Then, the superovulated female rat and adult male rat were allowed to mate, and only those females showing vaginal plugs next morning were recorded as ED 0.5 and kept for further embryo collection. On ED 4.5, the uterus was excised from the mated female rat and flushed with M2 medium (Sigma, M7167). The flushed blastocyst-cell stage embryos were then selected and transferred

into 200 μl drops of M2 medium supplemented with 0.5 mg/ml hyaluronidase (Sigma, H4272) for 3 min, washed by fresh M2 medium, transferred into 400 μl drops of M16 medium (Sigma, M7292), and kept in the $CO_2$ incubator (37°C, 5% $CO_2$, 95% air) for embryo transfer.

*Preparation of vasectomized male rats*

To increase the success rate of mating, adult male rats were first mated with female homosexual parabiotic pairs and then mated with the heterosexual parabiotic pairs that had not undergone UTx. Only those male rats that make the female parabionts of both homosexual and heterosexual parabiotic pairs with vaginal plugs were subjected to receive vasectomy (Fig. 3B). The sterility of vasectomized males was proven 2 weeks later (Fig. 3A).

*Embryos transferred to the heterosexual parabiotic pairs*

Approximately 8 weeks after UTx, the survival heterosexual parabiotic pairs were selected when the female parabionts were in proestrus and caged with a trained sterile male rat. On the following morning, only those female parabionts with vaginal plugs were proposed to receive embryo transfer. Three days later, blastocyst-cell stage embryos were transferred to both grafted uteruses of male parabionts and native uteruses of female parabionts. Briefly, the heterosexual parabiotic pairs were firstly anesthetized by isoflurane, and frontal incisions were made in the abdominal skin of parabiotic pairs. After separating the adhesive tissues, the native uterus of female parabiont and the grafted uterus of male parabiont were fully exposed. Under a dissecting microscope, small holes were made in the native uterus and the grafted uterus by using a needle. Then 6 to 10 blastocyst-cell embryos were transplanted into left uteruses of female parabionts, grafted uteruses of male parabionts, and right uteruses of female parabionts, respectively (Fig. S6A). The body wall and skin were finally sewed. A thermostatically heated pad was used to keep animal's body temperature during and after surgery until fully recovery from anesthesia.

*Exploratory laparotomy after embryo transfer*

Heterosexual parabiotic pairs were performed an exploratory laparotomy at 14 days after embryo transfer (ED 18.5). Under anaesthesia, abdomens of female and male parabionts were sectioned, adhesive tissues were carefully isolated, and both native uteruses and grafted uteruses were exposed. The numbers of normally developing embryos and absorbed embryos were counted, and then the abdominal walls were sutured.

**Caesarean and breast feeding**

To avoid labour pain, caesarean sections were performed on pregnant female parabionts and pregnant male parabionts under anaesthesia on ED 21.5. The native uterus in female parabiont was removed after ligating and cutting the bilateral uterine vessels and ovarian vessels. Fetuses were removed from the uterus, received a 37°C saline bath, and placed on a heated pad at 29-31°C for 2 hours. The placentae were also isolated from the uterus. At the same time, the grafted uterus and stoma in male parabiont were removed after ligating and cutting the right common iliac vessels above the cuff. Fetuses and placentae were treated in a similar manner. After closing the abdominal walls, the division surgery and antibiotic therapy (20 mg/kg ampicillin) were performed. 2 hours later, the number and status of fetuses were counted and recorded respectively, and all fetuses and placenate were weighted. The live fetuses were then fostered by surrogate mothers that had given birth to healthy litters within 24 hours.

**Spectral karyotyping**

Animals were anesthetized 3 hours after treatment with 100 μg/kg colchicine. After exposing femur bones and cutting off joint heads, bone marrow cells were collected by washing of 0.85% saline. Then the suspension was received hypotonic treatment with 0.075 mol/L KCL at 37°C, fixed in methanol-glacial acetic acid (3:1), and dropped onto cleaned wet slides. After dyeing with Giemsa solution (Sigma, 329757421), the morphological characteristics of chromosomes were acquired and analyzed by automatic scanning microscope and image analysis system (Leica, GSL-120) with minor artificial revisions (*22*).

**Statistics and image analysis**

Statistical analysis was performed using PASW Statistics 18.0 and graphs were

generated using Graphpad Prism 8.0. Student's T-test was used to compare the measurement data between two groups. The numbers of $CD8^+$ cells were estimated in 5 random fields for each animal and analyzed by Wilcoxon test. Chi-square test or Fisher exact test were used for proportions. Data were presented as mean±SD. A *p*-value of less than 0.05 was defined as statistically significant.

## SUPPLEMENTARY MATERIALS

**Movie 1.** A non-pregnant female parabiont (red mark) and a non-pregnant male parabiont (blue mark) at ED 18.5 after embryo transfer.
**Movie 2.** A pregnant female parabiont (red mark) and a non-pregnant male parabiont (blue mark) at ED 18.5 after embryo transfer.
**Movie 3.** A pregnant female parabiont (red mark) and a pregnant male parabiont (blue mark) at ED 18.5 after embryo transfer.
**Movie 4.** Surviving fetuses delivered from male parabionts at 24 hours after caesarean sections.

## Figures and figure legends

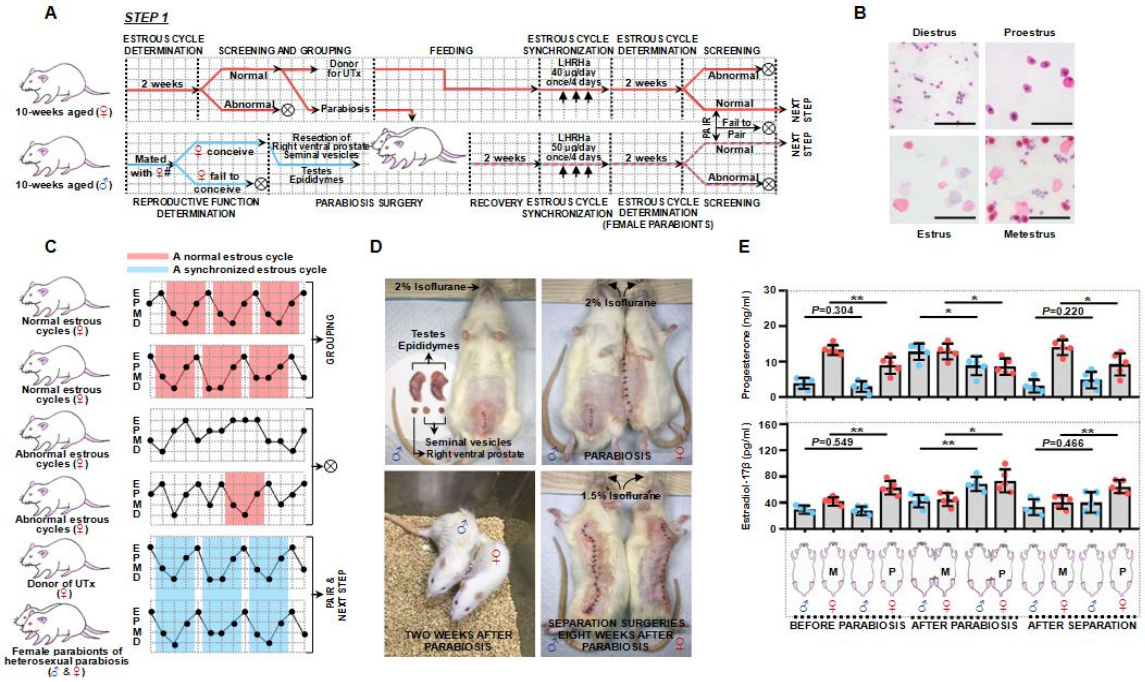



**Fig. 1. Modeling and screening heterosexual parabiotic pairs.** (A) Graph illustrating the progress of modeling and screening heterosexual parabiosis. This is the first step of our rat model of male pregnancy. Hashtag indicates superovulated female rats. Cross means rats were removed from the experiment. (B) Photomicrographs of vaginal smear from female rats in diestrus, proestrus, estrus and metestrus respectively. Scale bars, 100 μm. (C) Representative images exhibiting estrous cycles in female rats and female parabionts of heterosexual parabiotic pairs in 2 weeks. E, P, M, D represents estrus, proestrus, metestrus and diestrus. Cross means rats were removed from the experiment. (D) Procedure of heterosexual parabiosis surgeries and separation surgeries. (E) Serum levels of progesterone and estradiol-17β in selected heterosexual pairs before parabiosis surgeries, six weeks after parabiosis surgeries and two weeks after separation surgeries. n=5 per group. M means late stage of metestrus. P means late stage of proestrus. Error bars indicate SD. **P*<0.05, ***P*<0.01.

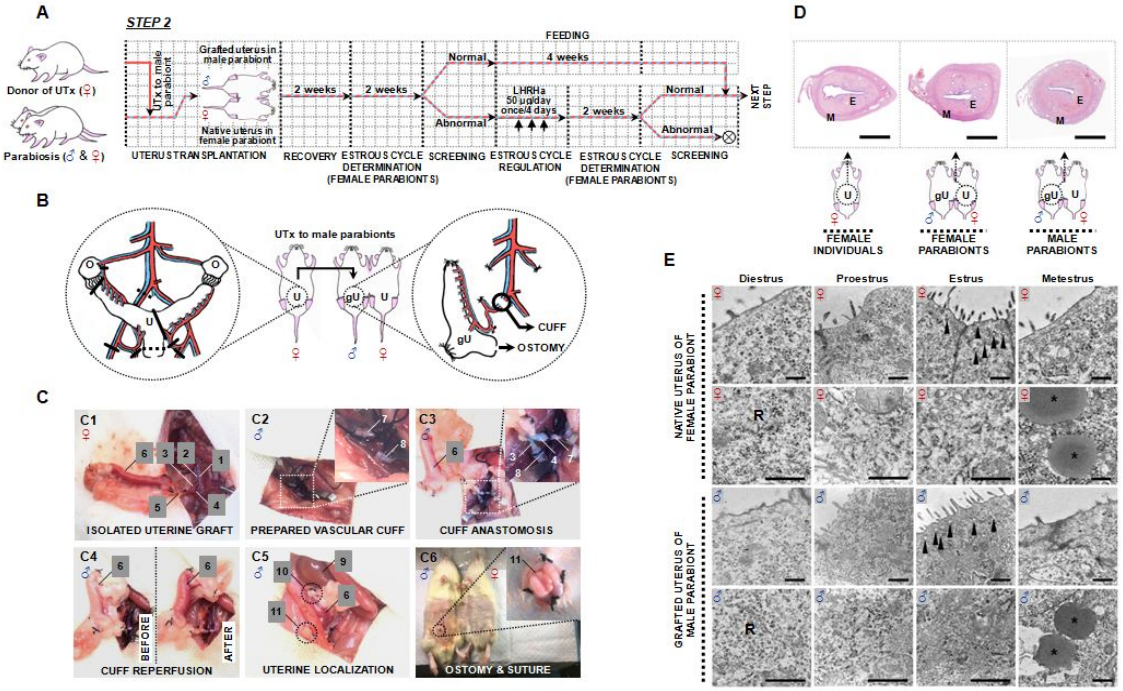



**Fig. 2. Uterus transplanted to male parabionts.** (A) Illustration of uterus transplantation (UTx) and recovery. This is the second step of our rat model of male pregnancy. Cross means rats were removed from the experiment. (B) Schematic drawing of UTx. U means uterus. gU means grafted uterus. O means ovary. Solid line represents ligation and cut. Dotted line represents cut. (C) Surgical procedure for UTx. 1=aorta; 2=vena cava; 3=common iliac artery; 4=common iliac vein; 5=uterine vessels; 6=uterus; 7=common iliac arterial cuff; 8=common iliac venous cuff; 9=kidney; 10=connective tissue under the kidney; 11=stoma. (D) H&E staining of native uteruses of female individuals, native uteruses of female parabionts and grafted uteruses of male parabionts (8 weeks after UTx). U means uterus. gU means grafted uterus. E means endometrium. M means muscle layer. Scale bars, 1 mm. (E) Electron microscopy of native uteruses of female parabionts and grafted uteruses of male parabionts. Diestrus, proestrus, estrus and metestrus were determined by vaginal smears of female parabionts (n=3 in each stage). R means free ribosomes. Arrowhead means secretory granules. Asterisk means lipid vacuoles. Scale bars, 500 nm.

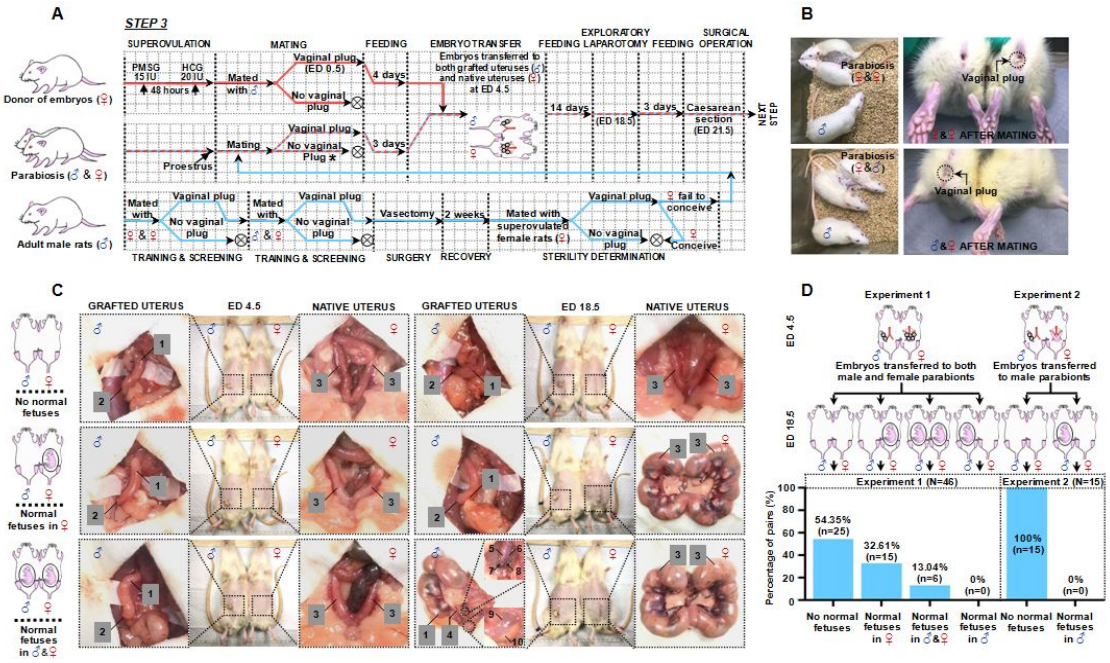



**Fig. 3. Transferred embryos developed in grafted uteruses of male parabionts under pregnant blood exposure from female parabionts.** (A) Illustration of embryo transfer procedure. This is the third step of our rat model of male pregnancy. Cross means rats were removed from the experiment. (B) Male rats were trained for mating with female parabionts before vasectomy. (C) Typical pictures of heterosexual parabiotic pairs, grafted uterus of male parabionts, and native uterus of female parabionts at ED 4.5 (before embryo transfer) and at ED 18.5 (exploratory laparotomy). 1=grafted uterus; 2=uterine stump close to stoma; 3=native uterus; 4=uterine vessels; 5=common iliac artery; 6=common iliac vein; 7=common iliac arterial cuff; 8=common iliac venous cuff; 9=bladder; 10=atrophied left ventral prostate. (D) Pregnancy rate of heterosexual parabiotic pairs at ED 18.5. Pregnancy means there is at least one normally developing fetus in native uteruses of female parabionts or in grafted uteruses of male parabionts.

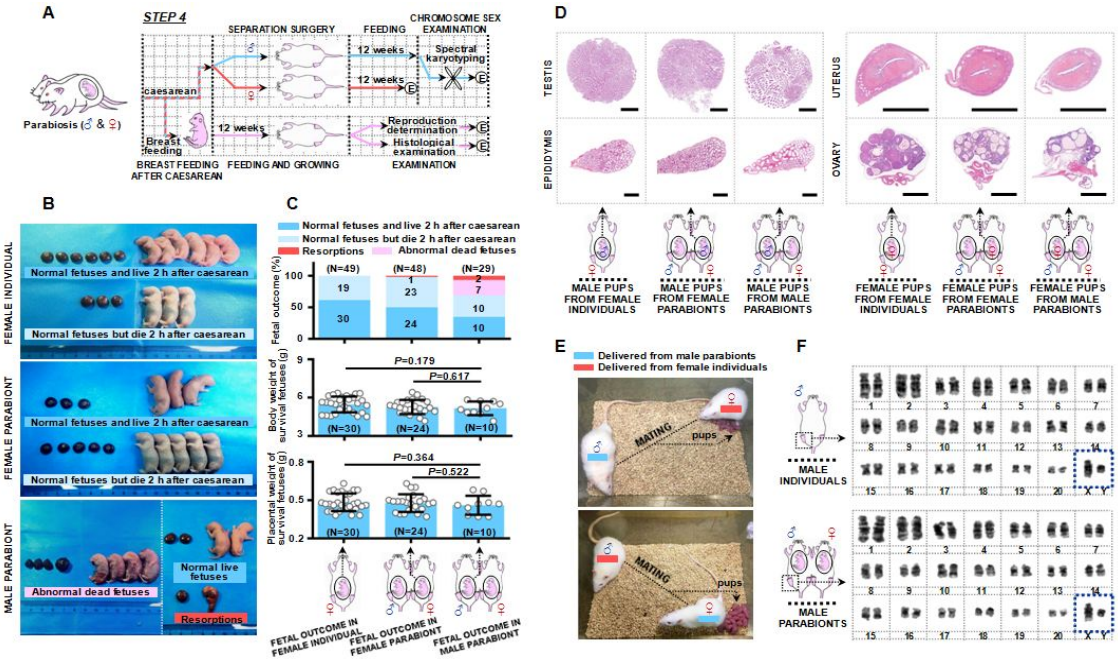



**Fig. 4. Surviving fetuses and male parabionts after caesarean sections.** (A) Illustration of experimental protocol after caesarean sections. This is the last step of our rat model of male pregnancy. E means euthanization. (B) Representative images of fetuses and placentas at 2 hours after caesarean sections from female individuals, female parabionts and male parabionts. (C) Fetal outcome after caesarean sections. The body weight and placental weight of surviving normal fetuses at 2 hours after caesarean sections were also exhibited. Error bars indicate SD. (D) H&E staining of testis and epididymis of male offsprings (12 weeks) and uterus and ovary of female offsprings (12 weeks). Scale bars=5 mm in testis and epididymis; scale bars=2 mm in uterus and ovary. (E) Male and female fetuses delivered from male parabionts developed to adulthood with reproductive competence. (F) Representative images of spectral karyotyping (femoral bone marrow). Male individuals refer to the castrated male rats (with reproductive competence before castration, received castration surgery at approximately 3 months old, sampled for karyotype analysis at approximately 12 months old). Male parabionts refer to those who had experienced successful male pregnancy and survived after separation surgeries (sampled for karyotype analysis at approximately 12 months old). Blue box means sex chromosome.

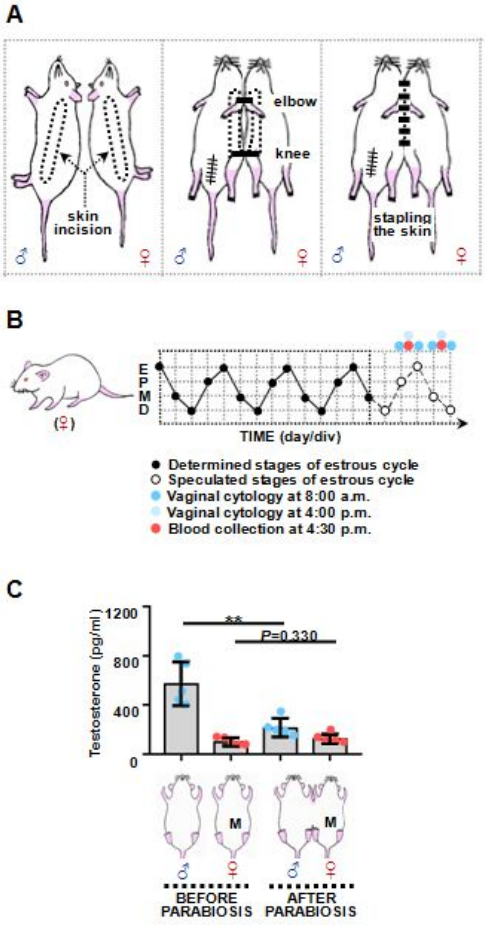
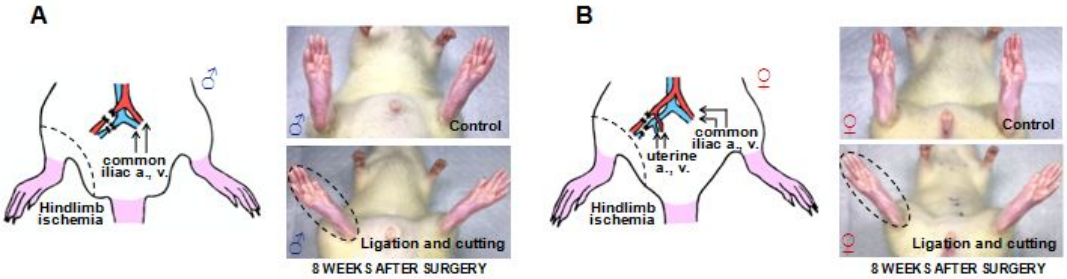



**Fig. S1. Parabiosis surgeries, blood collections and serum levels of testosterone.** (A) Illustration of heterosexual parabiosis surgeries. After resections of testes, epididymes, seminal vesicles and right ventral prostate, a skin incision was made along the opposing flanks. Then a connection was secured by suturing the elbows and knees using a suture pass through the soft tissue of each joint. Finally the ventral and dorsal skin was stapled respectively. (B) Illustration of blood collections in late stage of metestrus and in late stage of proestrus. A figure of female estrous cycles was drawn to speculate the approximate time of late stage of metestrus and late stage of proestrus. E, P, M, D represents estrus, proestrus, metestrus and diestrus. (C) Serum levels of testosterone in selected heterosexual pairs before and after parabiosis surgery. n=5 per group. M indicates late stage of metestrus. Error bars indicate SD. $^{**}P<0.01$.




C

FERTILITY AND PERINATAL OUTCOMES AFTER HINDLIMB ISCHEMIA

| OUTCOME | CONTROL | ISCHEMIA |
|---|---|---|
| Mating (1st) | 4/6 | 3/6 |
| Pregnancy | 2/4 | 2/3 |
| Mating (2nd) | 2/4 | 1/4 |
| Pregnancy | 1/2 | 0/1 |
| Total pregnancy rate | 3/6 | 2/6 |
| Survival mothers | 3/3 | 2/2 |
| PUPS | | |
| Live | 31/31 | 23/23 |
| Stillborns/death | 0/31 | 0/23 |
| Male | 15/31 | 12/23 |
| Female | 16/31 | 11/23 |

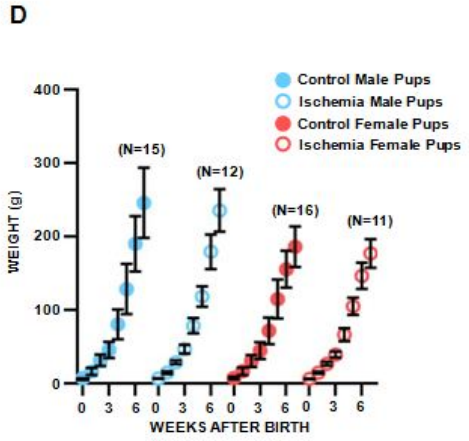



**Fig. S2. Effect of right common iliac vessel ligation and cutting on hindlimb ischemia and female fertility in Lewis rats.** (A and B) Schematic diagram illustrating ligation and cutting of right common iliac vessels in male and female rats. Right hindlimb (dotted circle) was observed at 8 weeks after the surgery. Control means not subjected to any surgical manipulation. (C) Female fertility and perinatal outcomes after ligation and cutting of right common iliac vessels. Mating represent successful mating determined by visible vaginal plugs, and those non-pregnant females at the first round had a second chance to mate. (D) Weight evolution of pups from different experimental groups.

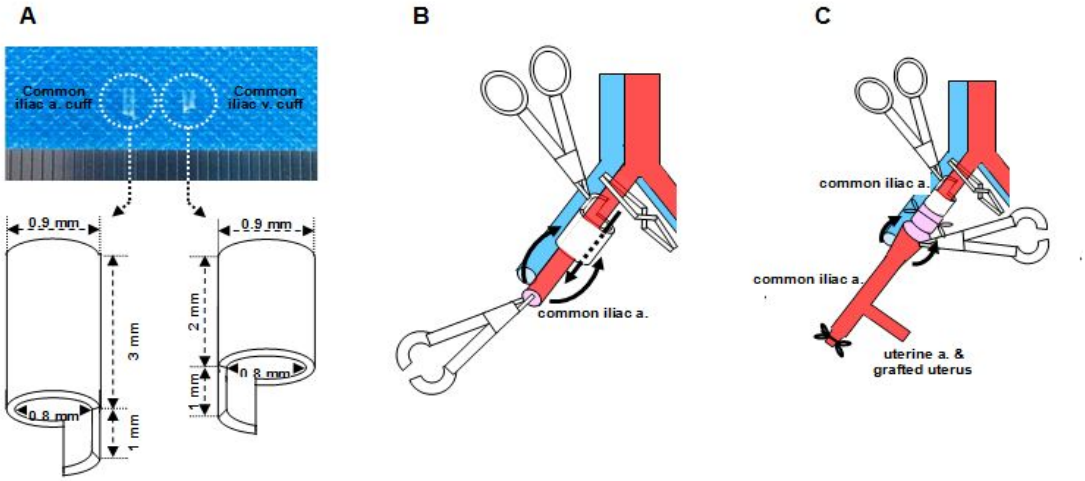



**Fig. S3. Cuff technique for establishing vascular anastomosis.** (A) Length and thickness parameters of vascular cuffs. (B) The first step of cuff technique is pulling the common iliac artery of male parabiont though the cuff. Then the artery was folded over the cuff and secured with a 8-0 nylon tie to expose the endothelial surface. The common iliac vein of male parabiont was treated in the same way. (C) The cuffed common iliac artery was inserted into the donor common iliac artery and secured with another 8-0 nylon tie and re-secured with a 5-0 suture. The cuffed common iliac vein of male parabiont was treated in the same way.

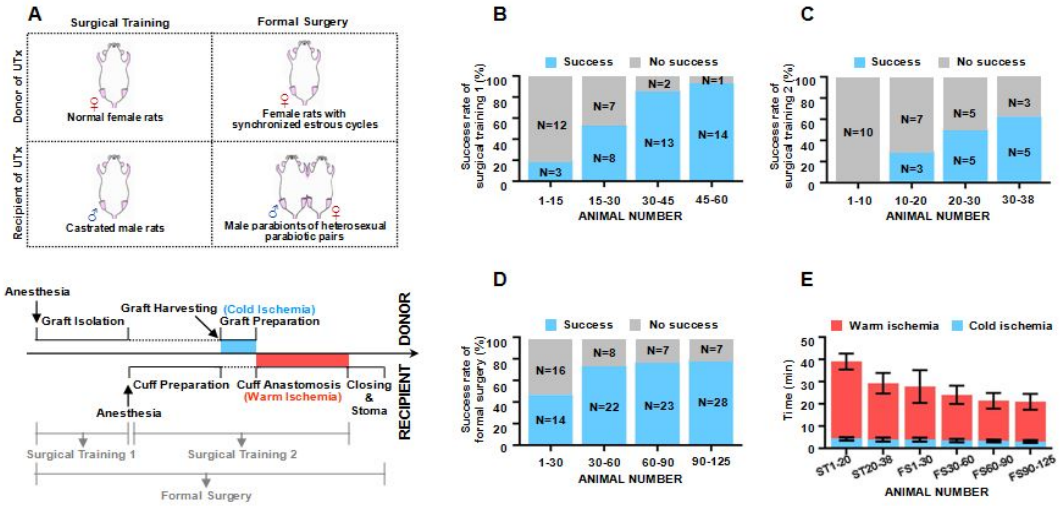
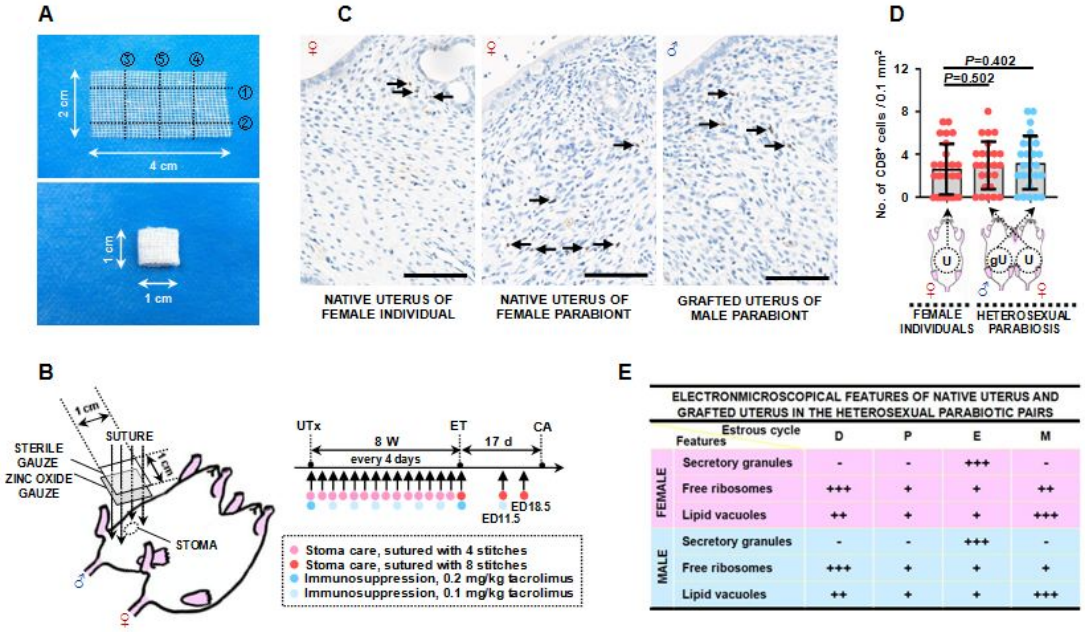


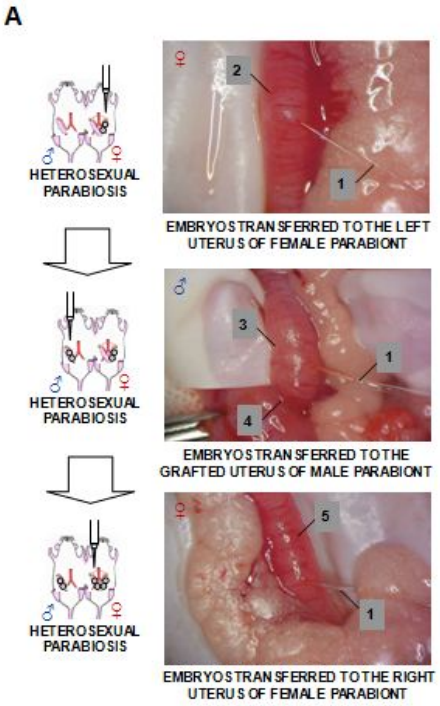

**Fig. S4. Success rate and graft ischemia time of surgical training and UTx formal surgeries.** (A) Illustration of surgical training 1, surgical training 2 and UTx formal surgeries. The processes of surgical training and UTx formal surgeries were performed similarly as shown in Fig. 2B and Fig. S3, but the donors and recipients were different. (B) Success rate of surgical training 1. (C) Success rate of surgical training 2. (D) Success rate of UTx formal surgeies. (E) Ischemia time of grafts in surgical training 2 and UTx formal surgeries. ST means surgical training 2. FS means UTx formal surgeries.




ELECTRONMICROSCOPICAL FEATURES OF NATIVE UTERUS AND GRAFTED UTERUS IN THE HETEROSEXUAL PARABIOTIC PAIRS

| | Features \ Estrous cycle | D | P | E | M |
|---|---|---|---|---|---|
| FEMALE | Secretory granules | - | - | +++ | - |
| | Free ribosomes | +++ | + | + | ++ |
| | Lipid vacuoles | ++ | + | + | +++ |
| MALE | Secretory granules | - | - | +++ | - |
| | Free ribosomes | +++ | + | + | + |
| | Lipid vacuoles | ++ | + | + | +++ |

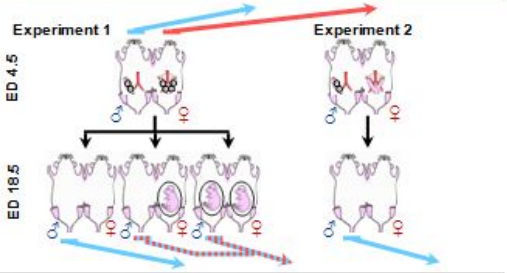
**Fig. S5. Stoma care, immunosuppression and uterine electronmicroscopical features after UTx.** (A) A 4×2 cm rectangular gauze was folded into a 1×1 cm square gauze according to the ordered black dotted line. (B) Protocols of stoma care (dressing change) and immunosuppression (tacrolimus injection) after UTx until caesarean sections. UTx means uterus transplantation; ET means embryo transfer; CA means caesarean section. (C) CD8 immunohistochemical staining of native uteruses of female individuals, native uteruses of female parabionts and grafted uteruses of male parabionts at 8 weeks after UTx. Arrows point to CD8 positive cells. Scale bars, 100 μm. (D) Density of CD8 positive cells. n=5 per group, and each animal was estimated in 5 random fields. U means uterus. gU means grafted uterus. (E) Major electronmicroscopical features of native uteruses of female parabionts and grafted uteruses of male parabionts at 8 weeks after UTx. n=3 in each estrous stage. D, P, E, M represents diestrus, proestrus, estrus, and metestrus determined by vaginal smears from female parabionts. The + symbol indicates the relative frequency of a feature; the - symbol indicates the total absence of a feature.




B

DEVELOPMENT OF TRANSFERRED EMBRYOS

| DEVELOPMENTAL PROGRESS | MALE PARABIONT | FEMALE PARABIONT |
|---|---|---|
| Number of transferred embryos at ED 4.5 | 280 (100.00%) | 562 (100.00%) |
| Number of fetal resorptions at ED 18.5 | 2 (0.71%) | 10 (1.78%) |
| Number of normal development fetuses at ED 18.5 | 27 (9.64%) | 169 (30.07%) |




C

DEVELOPMENT OF TRANSFERRED EMBRYOS IN MALE PARABIONTS

| DEVELOPMENTAL PROGRESS | NON-PREGNANT BLOOD EXPOSURE (EXPERIMENT 1) | PREGNANT BLOOD EXPOSURE (EXPERIMENT 1) | NON-PREGNANT BLOOD EXPOSURE (EXPERIMENT 2) |
|---|---|---|---|
| Number of transferred embryos at ED 4.5 | 135(100.00%) | 145 (100.00%) | 90 (100.00%) |
| Number of fetal resorptions at ED 18.5 | 0 (0.00%) | 2 (1.38%) | 0 (0.00%) |
| Number of normal development fetuses at ED 18.5 | 0 (0.00%) | 27(18.62%) *# | 0 (0.00%) |

**Fig. S6. Development of transferred embryos at ED 18.5.** (A) At ED 4.5, embryos were transplanted into left uteruses of female parabionts, grafted uteruses of male parabionts, and right uteruses of female parabionts, respectively. 1=embryo pipette; 2=left uterus of female parabiont; 3=grafted uterus of male parabiont; 4=uterine stump close to stoma; 5=right uterus of female parabiont. (B) Number of transferred embryos at ED 4.5, fetal resorptions at ED 18.5, and normal development fetuses at ED 18.5 in male and female parabionts. Experiment 1 means embryos transferred to both male and female parabionts at ED 4.5. Experiment 2 means embryos only transferred to male parabionts at ED 4.5. (C) Effect of pregnant blood exposure (Experiment 1) and non-pregnant blood exposure (Experiment 1 and 2) on development of transferred embryos in male parabionts. *$P$<0.001 compared with non-pregnant blood exposure (Experiment 1); $^{\#}P$<0.001 compared with non-pregnant blood exposure (Experiment 2).

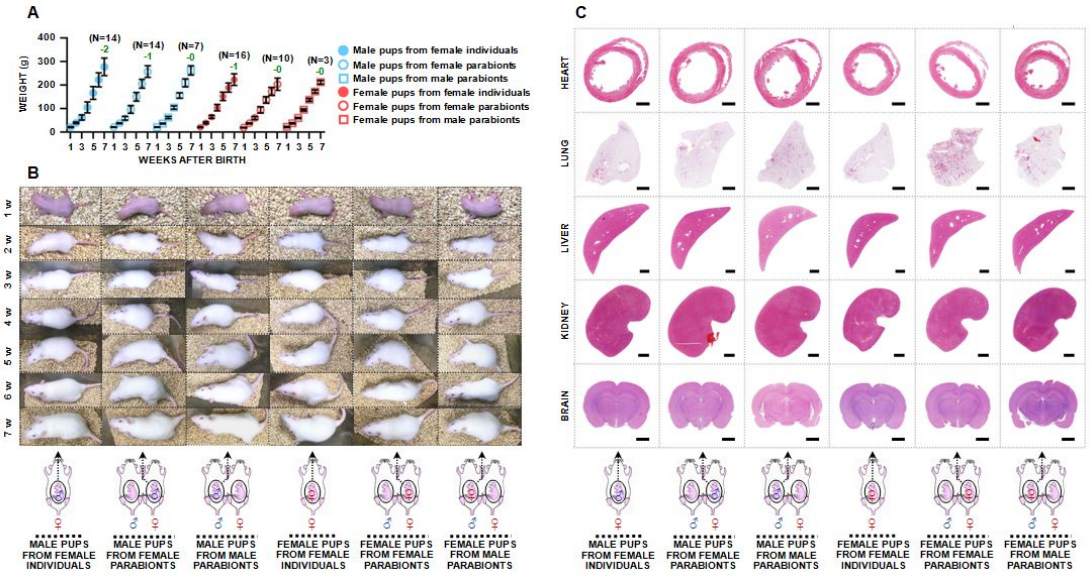



**Fig. S7. Growth of surviving fetuses after caesarean sections.** (A) Weight evolution of surviving fetuses. The green number represents the number of pups eaten by foster mothers during breast feeding. (B) General observations of surviving fetuses during growing up. (C) H&E staining of heart, lung, liver, kidney and brain of male and female offsprings (12 weeks). Scale bars=2 mm in each tissue.

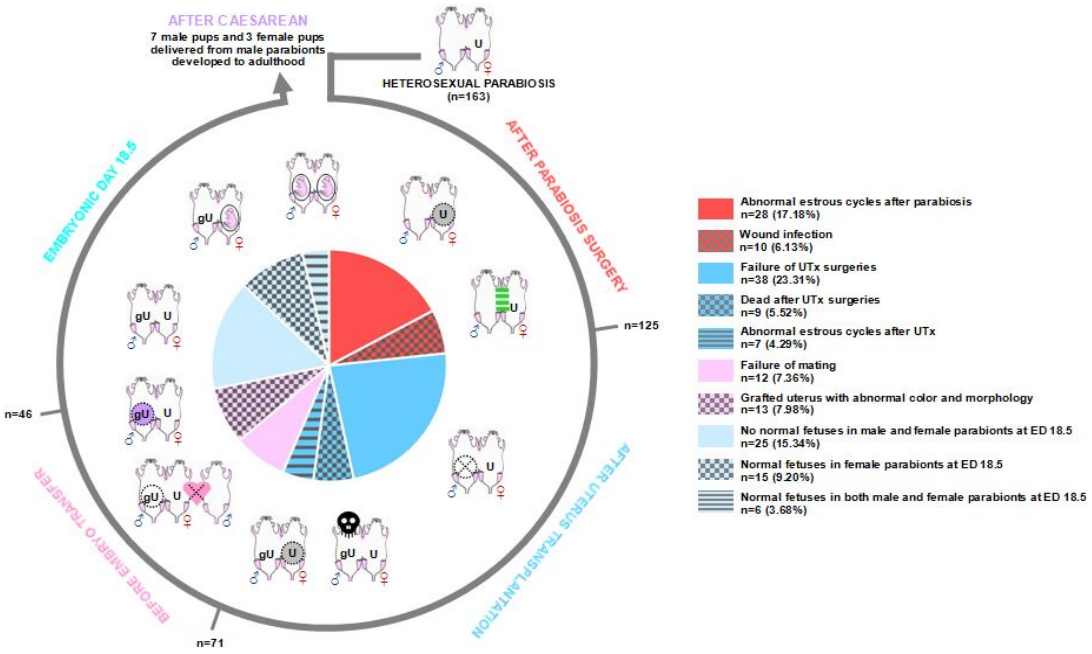



**Fig. S8. Imaging procedure of our rat model of male pregnancy.** A total of 163 heterosexual parabiotic pairs were proposed to achieve male pregnancy, and only 6 male parabionts succeed (3.68%). A total of 280 blastocyst-stage embryos were transferred to grafted uteruses of male parabionts at ED 4.5 (562 blastocyst-stage embryos were transferred to native uteruses of female parabionts for pregnant blood exposure), and only 27 normal fetuses were observed at ED 18.5 (9.64%). After caesarean sections, 10 pups were survived and develop to adulthood (3.57%).