

# Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61960R1
Full Title:	A syngeneic murine model of endometriosis using naturally cycling mice
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Additional Information:	
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**TITLE:**

A Syngeneic Murine Model of Endometriosis Using Naturally Cycling Mice

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**KEYWORDS:**

endometriosis, animal model, syngeneic, murine, retrograde menstruation, endometrium

**SUMMARY:**

Many rodent models of endometriosis are limited by technical complexity, reproducibility, and/or need for immunocompromised animals or special reporter mice. We present a simplified system of lesion induction using any experimental mouse with an independently verifiable, objective scoring system and with no requirement for ovariectomy or survival surgery.

**ABSTRACT:**

Endometriosis is a leading cause of pelvic pain and infertility. It is defined by the presence of endometrial tissue in extrauterine locations. The development of novel therapies and diagnostic

tools for endometriosis has been limited due in part to challenges in studying the disease. Outside of primates, few mammals menstruate, and none develop spontaneous endometriosis. Rodent models are popular but require artificial induction of endometriosis, with many utilizing either immunocompromised mice or surgically induced disease. Recently, more attention has been given to models involving intraperitoneal injection. We present a murine model of endometriosis that integrates several features of existing endometriosis models into a novel, simplified system that relies on microscopic quantification in lieu of subjective grading. In this model, we perform hormonal stimulation of donor mice, intraperitoneal injection, systematic abdominal survey and tissue harvest, and histologic quantification that can be performed and verified at any time after necropsy. This model requires minimal resources and training; does not require expertise by lab technicians in murine survival surgery or in the identification of gross endometriotic lesions; can be used in immunocompromised, immunocompetent, and/or mutant mice; and reliably creates endometriotic lesions that are histologically consistent with human endometriotic disease.

## **INTRODUCTION:**

Endometriosis is an enigmatic disease of the female reproductive tract with significant financial and health burdens on women<sup>1,2</sup>. The etiology of endometriosis is not completely understood, and multiple explanations have been proposed including coelomic metaplasia, embryonic Müllerian rests, recruitment of bone marrow derived progenitor cells, and retrograde menstruation<sup>3</sup>. While multiple aspects of these proposed mechanisms may be involved, and no single explanation can account for all forms of the disease, the leading model of endometriosis pathogenesis is retrograde menstruation. Retrograde menstruation is the passage of menstrual effluent through the fallopian tubes and into the peritoneal cavity; it is estimated that 90% of menstruating women regularly undergo retrograde menstruation<sup>4,5</sup>. Given this commonplace phenomenon of retrograde menstruation, why endometriosis develops only in a subset of women is unclear<sup>5</sup>. To better understand the etiology of this disease, direct human studies are not feasible and animal studies are warranted.

Endometriosis is a challenge both to treat and to study. Prevalence of the disease is not known but estimated to be 10%<sup>1</sup>. While some advanced types of endometriosis may be accurately identified through noninvasive imaging, a definitive diagnosis is only achieved through histopathological analysis of surgically obtained biopsy specimens; lesions that visually appear to be diseased, may in fact be fibrosis or scarring from other causes<sup>6</sup>. Severity and extent of disease does not correlate with symptomatology<sup>7</sup>.

Endometriosis lesions consist of heterogeneous cell types and populations which interact in complex ways within the microenvironment, therefore, limiting the usefulness of cellular models<sup>8,9</sup>. In vivo models exist, but these have inherent challenges and limitations<sup>10-12</sup>. Primate models are ideal but are often not feasible<sup>13-15</sup>. Few non-primate mammals menstruate and develop endometriosis spontaneously<sup>16</sup>. Rodent models of endometriosis exist but each have limitations<sup>17</sup>. Many of these models require survival surgery to suture or implant endometrial tissue into the donor recipient wall or bowel, adding technical complexity, need for anesthesia, and confounding immune factors from the surgery itself<sup>18-20</sup>. In addition, many models require ovariectomy and estrogen supplementation; while increasing lesion yield, this adds time,

expense, and additional survival surgery. Intraperitoneal (IP) injection models do not require anesthesia or survival surgery, and these models logically simulate retrograde menstruation better than suturing models<sup>21–23</sup>. Most IP models, however, are subject to more variability in lesion location due to the random dispersal of endometrial fragments following injection and therefore to more bias in lesion identification and measurement.

Here we present a murine model of endometriosis that integrates several features of existing endometriosis models into a novel, simplified, and efficient system that relies on microscopic quantification in lieu of subjective grading.

## **PROTOCOL:**

NOTE: The use of animals in this study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic Lerner Research Institute. All publicly available animal care and use standards were performed following guidelines by the National Institutes of Health.

### **1. Preparation of donor and recipient mice (see Figure 1)**

1.1. Ensure that appropriate approval is in place to work with laboratory animals.

1.2. Determine the strain of mouse. In these experiments, wildtype and mutant mice having a C57BL/6J background were utilized, but investigators using similar models report success with other mouse strains such as BALB/c mice<sup>10</sup>. In addition, donor and/or recipient mice can utilize reporter systems, such as GFP or RFP mice (see **Figure 2** and **Figure 3**).

1.3. For timing of the endometrial tissue transplant, ensure that the donor mice are between 22–24 days old at the time of gonadotropin injection. This ensures that they are reproductively naïve, e.g., not yet begun estrous cycling.

1.4. Ensure that the recipient mice are reproductively intact (no prior ovariectomy) between 2 to 4 months of age. While not required, it is recommended to place urine-soaked bedding from a male mice cage periodically in the recipient's cage to facilitate ongoing estrous cycling and again at 72 h prior to endometrial tissue transplant.

NOTE: This will not ensure synchronization of estrous cycle of the recipients, as the placement urine-soaked bedding is highly dependent on the amount of male bedding used and has variable results. If synchronization of the estrous cycle is desired, three consecutive subcutaneous injections of 100 ng/100  $\mu$ L estradiol will bring all animals to the estrous phase. While we found no difference in lesion induction based upon phase of estrous cycle, other groups have found the cycle phase to be important<sup>10</sup>.

1.5. Subcutaneously inject Pregnant Mare Serum Gonadotropin (PMSG, 2 IU diluted into 200  $\mu$ L) into donor mice subcutaneously using a fine needle (25–27 G recommended). Do not give



PMSG to recipient mice, as it will trigger ovulation and subsequent high progesterone environment, which is less receptive to endometriosis formation.

NOTE: Prior mouse models have utilized either PMSG or estrogen to stimulate endometrial proliferation in the donor mice prior to endometrial harvest<sup>23</sup>. PMSG is recommended for the following reasons: The half-life of PMSG is 40 h whereas the half-life of 17-beta-estradiol is only 2 h. Utilizing a single subcutaneous injection of PMSG in the donors 40 h prior to procurement of endometrial tissue provides a sustained duration of exposure to gonadotropin stimulation, which acts to stimulate endogenous estrogen. Many preparations of exogenous estrogen require multiple injections to adequately prime the endometrium.

1.6. Following PMSG injection, schedule necropsy, procurement, and transplant into the recipient between 38–42 h. Time the endometrial tissue harvest following gonadotropin injection to ensure collection of tissue before ovulation, which occurs at 42 h post injection. Ovulation produces a high progesterone (P4) environment, which would reduce lesion establishment.

## **2. Procurement of donor mouse endometrial tissue**

2.1. After euthanasia of donor mouse (using CO<sub>2</sub> chamber followed by cervical dislocation), spray the abdomen with 70% ethanol solution; this serves to reduce contamination of the procured tissue from skin flora and from dislodged hairs. With dissecting scissors, make a shallow transverse snip at midline through the skin and subcutaneous tissue. Then grasping each side of the skin incision, use blunt traction to open the abdomen.

2.2. Identify the uterus. Before removing the uterus, trim away adjacent connective tissue. Transect each uterine horn just below their respective fallopian tube and then transect the cervix to remove the entire uterus en bloc.

2.3. After removal from the abdominal cavity, inspect the uterus carefully and remove any additional peripheral fat or connective tissue. Place the uterus in a droplet of cold PBS on a Petri dish. Determine and document the combined mass of the entire uterus.

2.4. Transect each horn from the uterus fundus, making the transection as close to the fundus as possible so as to maximize the length of each horn. Using the aid of a dissecting microscope, place one blade of the dissecting scissors inside the lumen of the first horn, then cut along the major axis of the tube. Carefully open the tube, keeping in mind which side is the serosa and which side is the epithelium.

2.5. Put 500 cc of saline or PBS on a new Petri dish; the liquid will stay together due to surface tension.

2.6. Then, perform fragmentation of the uterus in a uniform manner. It is better to have fewer larger lesions than many smaller lesions. Begin by separating off the epithelium from the myometrium by grasping the endometrial layer and peeling it away.

2.6.1. Alternately, simply fragment the tissue without separating off the myometrium (as long as the epithelial side is fully exposed), but retaining the myometrium lessens the physiologic relevance of this model to human disease. Ensure that fragments are as large as possible but small enough to pass through an 18 G needle; (1 mm x 1 mm is recommended).

2.6.2. Collect 10–12 of these 1 mm x 1 mm fragments from the first horn (which roughly corresponds to 40 mg tissue in the C57BL/6J mice). Place collected fragments into the liquid collection.

2.7. Perform the same steps for the other uterine horn for a total of about 24 fragments per mouse. Document the total number of fragments.

### **3. Peritoneal injection of tissue fragments into recipient mouse**

3.1. Aspirate the suspended 1 mm x 1 mm fragments using the blunt end of a 1 cc syringe; the total volume should be 1 mL.

3.2. Attach an 18 G needle to the full syringe and load the fluid into the needle. Consider a mock injection back into the Petri dish to ensure that all the tissue will pass through the needle.

3.3. Take the recipient mouse. Either before or after IP injection, obtain a vaginal smear of the recipient mouse for estrous cycle documentation (10  $\mu$ L of saline using the bulb syringe at the vaginal orifice and plated on the glass slide with the coverslip).

3.4. Perform intraperitoneal injection of the fragments with the syringe at a 45 degree angle, taking care to not inject subcutaneously.

3.5. If fragments remain after injection, draw an additional 200  $\mu$ L of the fluid into the syringe for injection to ensure that all the fragments are successfully injected intraperitoneally.

3.6. Once assured of no bleeding or complications, place the recipient mice back in their cages and feed a normal diet.

### **4. Harvest of endometriotic lesions**

4.1. Euthanize recipient mice at approximately 21 days following fragment injection post-transplant.

NOTE: From experience and from discussion with collaborators using similar models, the maximum lesion size and number occur at approximately 3 weeks post-transplant; after 3 weeks, lesions begin to regress in size.

4.2. Following euthanasia and cervical dislocation, spray the animal's abdomen with 70% ethanol and tent the skin to cut superficially with dissecting scissors. Incise the skin and subcutaneous space with scissors to bluntly open the abdomen.

4.3. Before any further dissection is undertaken, a complete survey for gross lesions is performed, with size measured by calipers and documented as to their anatomical region (see below).

4.4. Perform complete collection of three distinct anatomic regions en bloc (regardless of whether or not lesions can be appreciated grossly). If lesions are seen in other regions (e.g., bowels), these should be ignored and not collected unless the lesion traverses one of the three areas below.

4.4.1. A = abdominal wall/peritoneum (can either be flattened into a cassette or rolled up, as long as consistent between samples).

4.4.2. B = pancreas and mesenteric fat.

4.4.3. C = parauterine connective tissue and fat (white glistening tissue that surrounds the uterus but does not involve any organs other than the bladder; take care to not mistake the bladder for a lesion).

4.5. Place each dissected area in a cassette, appropriately labeled, and place it into formalin and process as per lab protocol for histologic sectioning.

4.6. Section formalin blocks in two slides (D1 and D2) per tissue area at two uniform depths.

## 5. Scoring of endometriotic lesions

5.1. Scan (at 40x magnification) and archive slides.

5.2. Use digital slide reading software, define the longest distance (X) between edges of an endometriotic lesion—whether the edge consists of glands or stroma—and mark it. A continuous lesion is defined by glands surrounded by stroma; the line does not necessarily traverse only endometriotic tissue (as in the case of determining endpoints on an irregularly shaped or undulating focus of endometriosis) but the two end points need to be connected by continuous stroma and/or glands (see **Figure 4**).

5.3. Make a second line (Y) 90 degrees across the first line, with the length of the second line determined following the rules above.

5.4. If multiple non-contiguous lesions are encountered, give each their own X and Y measurements.

263 5.5. Calculate the final score for each slide as the summation of areas (X\*Y) of each lesion.

264  
265 5.6. Take the larger of the scores from the two slides (D1 vs D2) as the final score for that  
266 region (A, B, C).

267  
268 5.7. Total the scores from each region to give the final microscopic score for that animal.

#### 269 REPRESENTATIVE RESULTS:

270  
271 For an initial proof of concept experiment, donor endometrium from RFP mice was injected into  
272 wildtype recipient mice. H&E staining revealed histopathologic confirmation of classic  
273 architecture of endometriosis lesion (**Figure 3A**). Fluorescent microscopy confirmed that the  
274 observed lesion in question originated from the donor (**Figure 3B**).

275  
276 The second experiment was performed using 10 wildtype C57BL/6J donors and 10 recipients. An  
277 additional 5 recipients received sham treatment (injected with PBS and not endometrial  
278 fragments) and assigned a random identification number; reviewers were blinded prior to  
279 necropsy and histopathologic review.

280  
281 All recipients received donor endometrial tissue within 42 h of PSMG donor treatment. There  
282 was no correlation between this time interval and final uterine weight or lesion size. At the time  
283 of donor procurement, average total uterine weight was  $54 \pm 9.5$  mg. Average fragment number  
284 was  $22.4 \pm 5.2$ , resulting in average fragment weight of  $2.5 \pm 0.5$  mg. Endpoint surgery occurred  
285 at either day 20 or day 22 for all recipients.

286  
287 With an average number of lesions of 1.5 and average gross total lesion diameter of 3.7 mm,  
288 these end points are similar to other studies utilizing mouse models with a similar weight of  
289 injected endometrial fragments (**Figure 2**)<sup>10</sup>. The prevalence of lesions for all mice was 80%. The  
290 mice without lesions had significantly greater endometrial fractioning compared to mice with  
291 lesions (30.5 total fragments versus 20.3, respectively; average total fragments for all mice was  
292 22.4) resulting in below average fragment size at the time of injection (1.9 mg vs 2.6 mg). As  
293 expected, sham controls (injected with saline and not endometrial fragments) had no gross or  
294 microscopic disease. In a subsequent study using an expanded number of mice, estrous phase of  
295 the recipient mouse was not associated with lesion number or microscopic disease score.

296  
297 Gross lesion number appears to be a poor surrogate marker for total lesion burden, as there was  
298 discordance between the macroscopic and the microscopic disease present in the tissues. In 60%  
299 (n = 6) of the cases, there was agreement between the macroscopic and microscopic scoring  
300 (agreement defined as both showing presence or absence and difference in total size was within  
301 3 mm). However, in 40% (n = 4) of the cases, there was disagreement, with 10% (n = 1) of the  
302 time macroscopic disease was absent but microscopic disease was present by histology, 10% (n  
303 = 1) macroscopic disease was seen despite no absence of endometriosis on confirmatory  
304 histology (**Figure 5**), and the remaining 20% (n = 2) there was agreement in presence but not  
305 magnitude of lesion size. Thus, macroscopic examination for lesions alone is not sufficient for the  
306 quantification of endometriosis disease burden.

## FIGURE AND TABLE LEGENDS:

### Figure 1: Overview of the model.

**Figure 2: Surveyed regions for lesion quantification.** While the bowel and other intraperitoneal locations can harbor lesions, it is more challenging to distinguish these grossly and an exhaustive survey of the abdomen would be more time intensive with diminishing returns. Therefore, a consistent, systematic approach of harvesting the complete tissue (regardless of whether gross disease is present) is performed in the three most common regions of endometriosis formation: the abdominal wall/peritoneum (**A**), the pancreas and mesenteric fat (**B**), and the parauterine fat (**C**). Labeled are representative images of microscopic findings of lesions from each of the three regions. 40x magnification.

**Figure 3: Induction of endometriosis using donor mice endometrium expressing red fluorescent protein.** (**A**) H&E section of the lesion. (**B**) Fluorescent microscopy with DAPI staining. 40x magnification.

**Figure 4: Representative images of the software used to measure the dimensions of the lesion dimensions and quantify lesion burden.**

**Figure 5: Representative samples of gross “lesions” that are not endometriosis by histopathologic examination.** 40x magnification.

## DISCUSSION:

Our study demonstrates that endometriosis can be reliably induced in mice without requiring use of ovariectomy and/or survival surgery, and that ectopic endometrial lesions can be identified and quantified using a standardized survey of the abdomen and histologic analysis.

Many murine studies of endometriosis utilize surgically induced endometriosis in which donor endometrium is sutured in place to the bowel, abdominal wall, or other intraperitoneal location<sup>12,20</sup>. This has the advantage of standardizing the size and location of the transplanted tissue. However, in addition to added logistical challenges of survival surgery, this may introduce confounding variables from the surgery itself, given that endometriosis is an inflammatory disease and that in a clinical setting, endometriosis typically develops prior to any surgical procedure. Intraperitoneal injection, on the other hand, more accurately models the retrograde menstruation that is thought to cause the majority of endometriosis lesions.

Ovariectomy is often performed in rodent models to reduce variability introduced by the estrous cycle; and as endometriosis is a hormone-dependent disease, supraphysiologic doses of estradiol are then administered in these cases. This practice arguably limits the applicability of a mouse model to human disease. Our study demonstrates that ovariectomy is not necessary to reliably create endometriosis lesions and that phase of estrous cycle does not impact the ability to establish lesions.

A shortfall of other intraperitoneal injection models is the reliance on subjective measures of lesion size or burden. While use of calipers or other instruments may provide objective measures, these measurements are recorded at the time of lesion harvest and therefore not able to be verified later and may be subject to intra-observer variability. In our model, histologic quantification can be performed and verified long after necropsy, meaning that those who perform the necropsy do not need to have expertise in identification of gross lesions and are more easily blinded as to the intervention received. Furthermore, as our work illustrates, a proportion of disease may be missed when only relying on gross disease; in addition, many suspected lesions may actually be physiologic (e.g., lymphoid tissue) or fibrosis. Finally, taking standardized sections of entire anatomic regions in each mouse further reduces variability. This approach of procuring the same amount of tissue per mouse prevents the inevitable underscoring that would occur with small samples and the over-scoring of large samples, especially since microscopic disease may be present.

Ultimately, these refinements serve to both standardize and simplify the approach, resulting in a mouse model that can study endometriosis in a high throughput fashion. This model is an assayable method to screen for pathways and drug targets, and our lab is currently utilizing this model for a drug validation study. This model is particularly helpful when attempting to determine the relative importance of aberrant gene expression (or drug treatment) in the donor endometrium versus the recipient peritoneal environment. It also can also be utilized with reporter mice (as we have shown) and immunocompromised and knockout mice.

In summary, we provide several important innovations that provide a murine model with high reliability, reproducibility, and objectivity in studying endometriosis. Our approach advances the field by not only providing a straightforward, streamlined process for lesion induction but also a standardized way of measuring and reporting data.

#### **ACKNOWLEDGMENTS:**

We would like to thank the members of the Reizes laboratory for their critical review and insights during preparation of the manuscript, as well as the Imaging and Histology cores at Lerner Research Institute for their assistance in data collection and data analysis. This work was supported through an internal grant funding through the Research Program Committee at Cleveland Clinic and by an external grant through the Society for Reproductive Investigation and Bayer. Research in the Reizes Laboratory is also funded through VeloSano Bike to Cure, Center of Research Excellence in Gynecologic Cancer, and through The Laura J. Fogarty Endowed Chair for Uterine Cancer Research. Cleveland Clinic owns the copyright permission for Figure 1 and Figure 2.

#### **DISCLOSURES:**

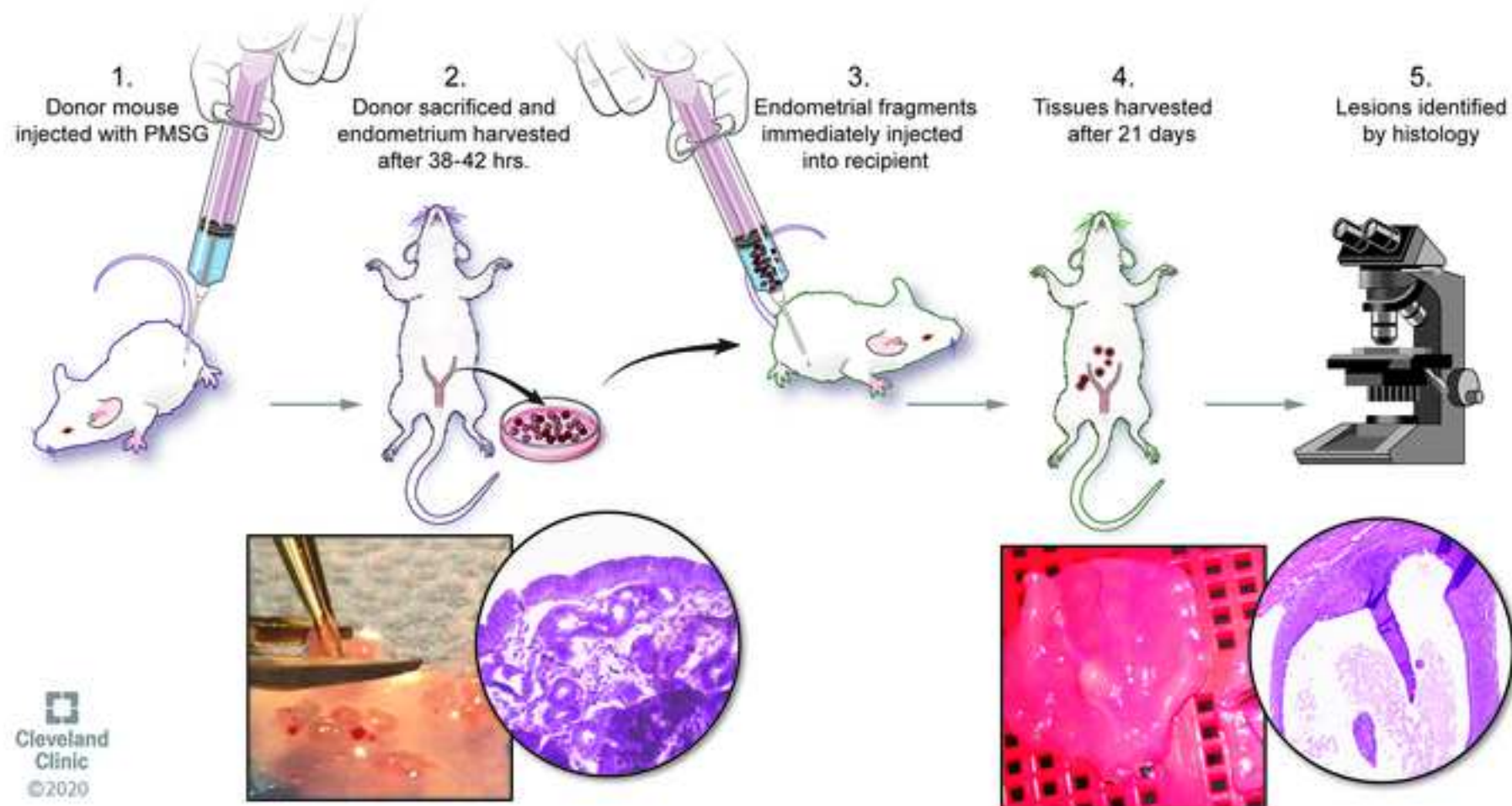
The authors have no conflicts of interest to disclose.

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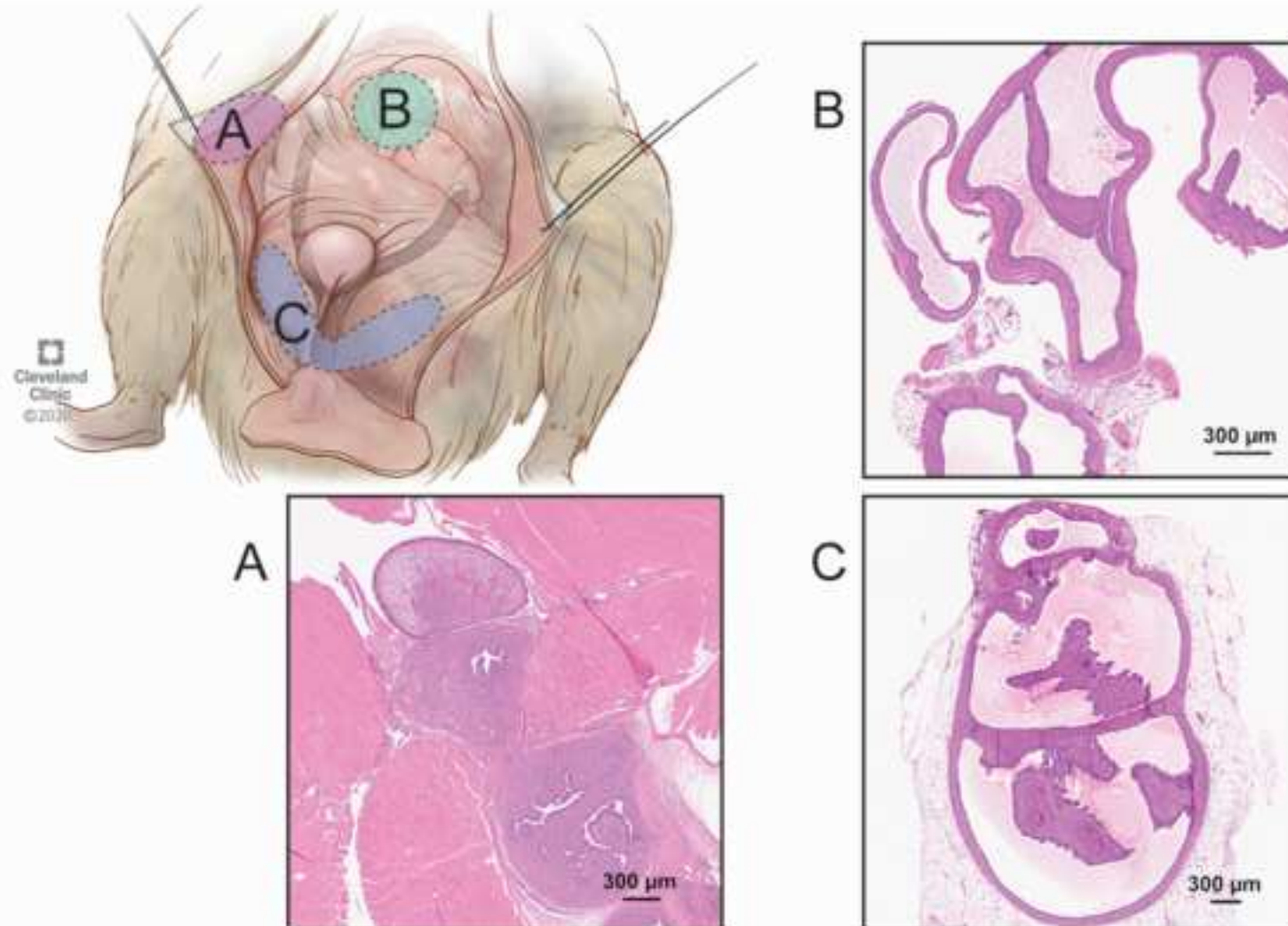
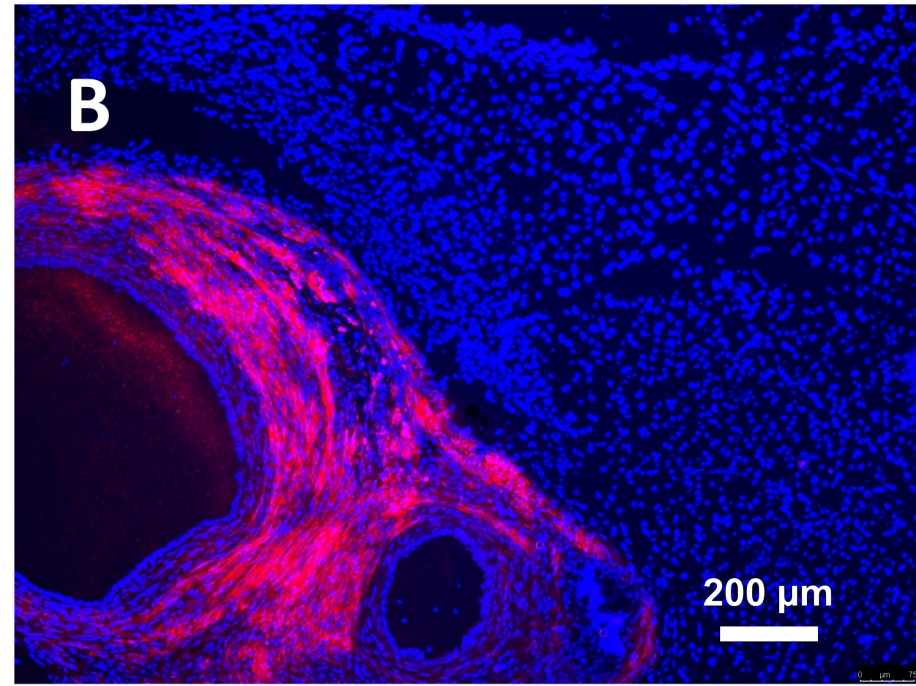
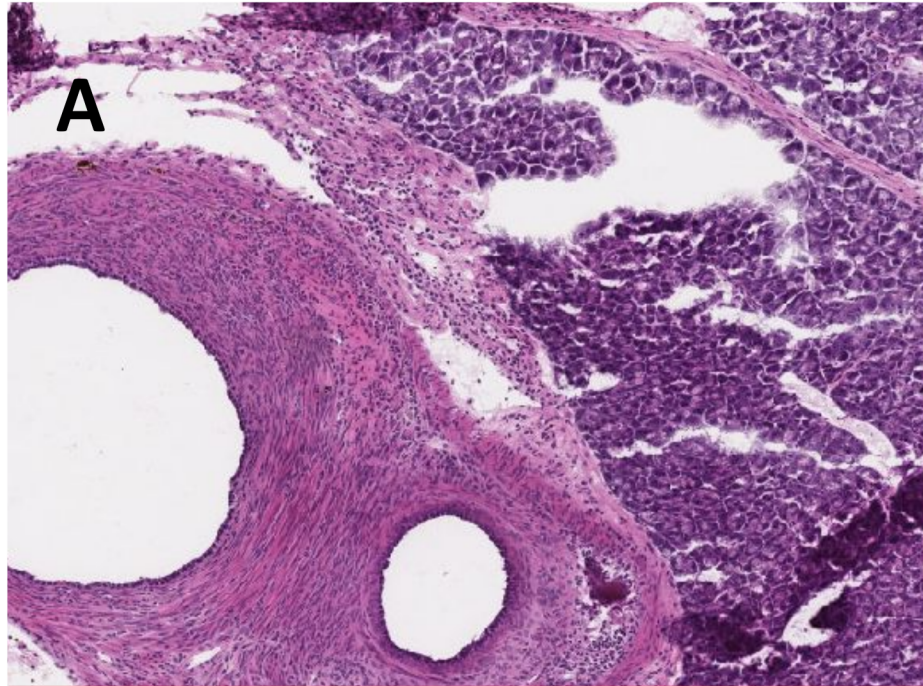




Figure 3

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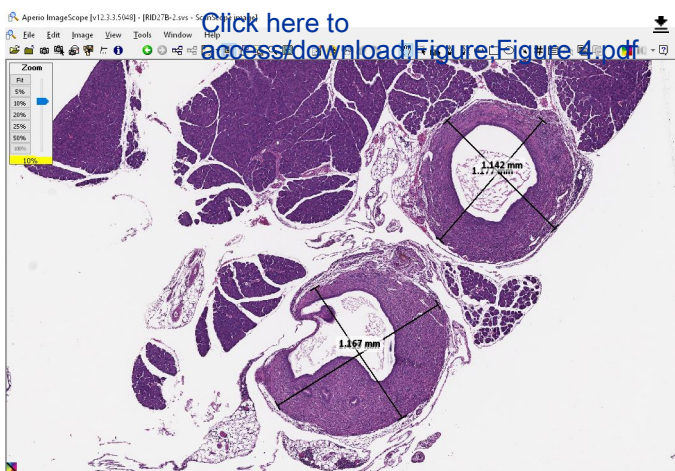
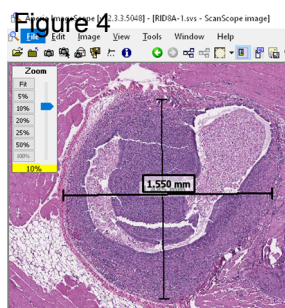
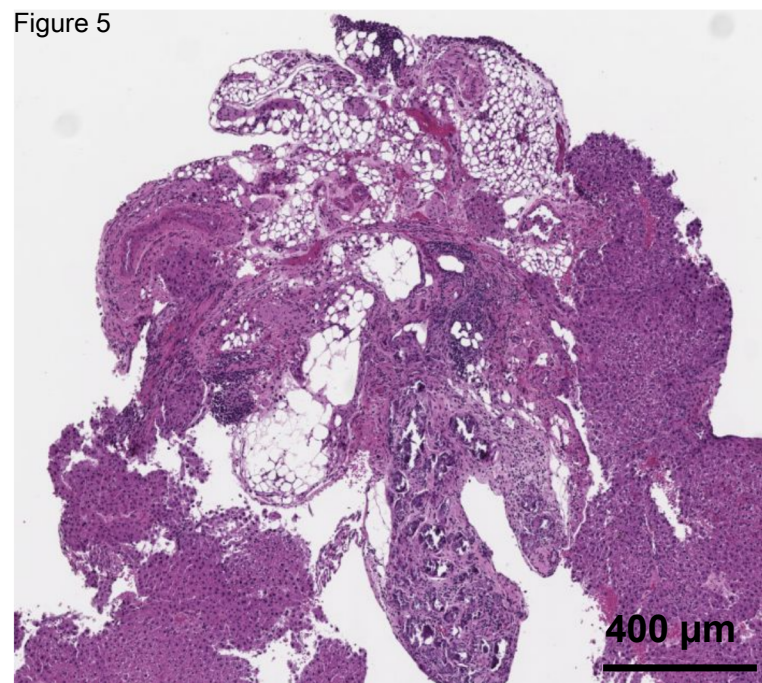
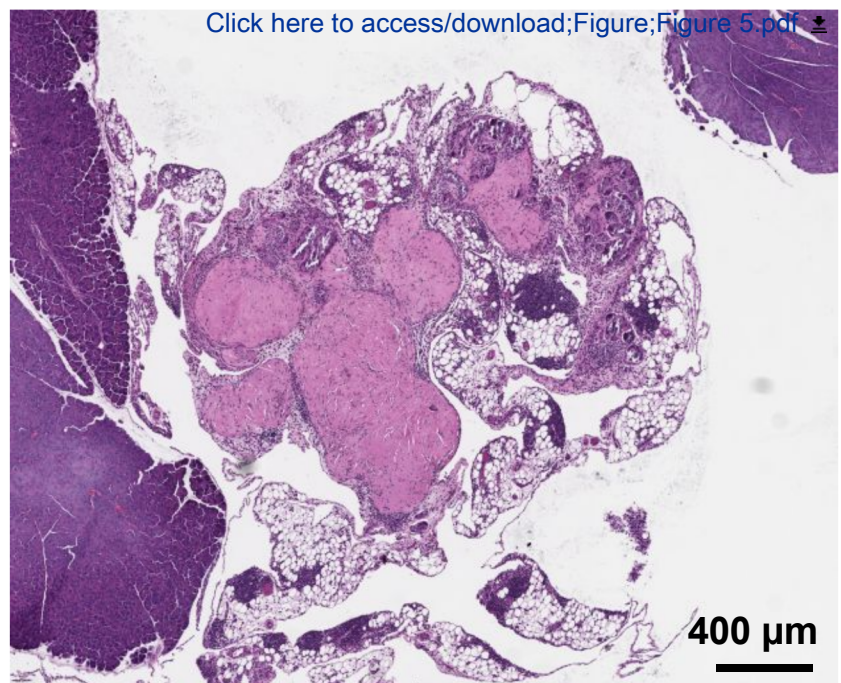




Figure 5



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Name of Material/Equipment	Company	Catalog Number	Comments/Description
<b><i>Supplies for injecting PMSG into donor mouse</i></b>			
1 mL Tuberculin syringe with 27G needle	Fisher Scientific	14-826-87	
Pregnant mare serum gonadotropin	Sigma-Aldrich	9002-70-4	
<b><i>Supplies for necropsy of donor mouse and tissue processing</i></b>			
6” serrated forceps, curved tip	Electron Microscopy Sciences	72993-6C	
70% ethanol solution	Pharmco	33000HPLCCS4L	70% solution dilute ethyl acetate 200 proof
Analytical balance	Mettler Toledo	ME54TE	
Carbon dioxide	TriGas Supplier		
Dissecting tray	Fisher Scientific	S14000	
No. 10 disposable scalpel	Fisher Scientific	NC9999403	
Scissors, curved	Electron Microscopy Sciences		72941
Scissors, straight	Electron Microscopy Sciences		72940
Stereo microscope	Leica Microsystems	Leica SE 4	For tissue dissection
Sterile phosphate buffered saline (PBS)	Institutional core facility supplies		
Surgical instrument sterilization tray	Electron Microscopy Sciences	66112-02	
Tissue culture dishes	Fisher Scientific	08-772E	
Weighing dishes	Fisher Scientific	02-202-103	
<b><i>Supplies for injecting into recipient mouse</i></b>			

1 cc syringe	BD Biosciences		301025	
18 G needle	Fisher Scientific	148265d		
200 uL pipette tip	Fisher Scientific	02-707-422		
Double distilled water	Institutional core facility supplies			
Latex bulb	Fisher Scientific	03-448-21		
Micro cover glass slip	VWR	48366-067		
Microscope slide	Fisher Scientific	12-544-7		
Standard light microscope	Leica Microsystems	DM IL		For evaluating vaginal cytology smears
<b><i>Supplies for harvesting tissue from recipient mouse</i></b>				
10% Buffered formalin	Fisher Scientific	SF100-4		
Biopsy foam pads	Fisher Scientific	22-038-222		
Precision Digital Calipers	Electron Microscopy Sciences	62065-40		
Processing/embedding cassettes	Fisher Scientific	22-272416		

### **Editorial comments:**

**We would like to thank the editors for consideration of our manuscript for publication. We have been incorporated the recommended edits as laid out below.**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**A thorough review of manuscript for spelling and grammatical errors has been performed by multiple authors.**

2. Please include a scale bar on all figures.

**This has been added.**

3. Please revise the title for conciseness. “that provides high reliability, reproducibility, and improved objectivity” can be removed.

**The title has been revised as suggested for conciseness. Thank you for this recommendation.**

4. Please specify how euthanasia is performed.

**The details of euthanasia has been added to the protocol portion of the manuscript.**

5. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

**This has been done.**

6. Please specify a journal section for the article. I would suggest Medicine.

**This has been done.**

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### **Reviewers' comments:**

**Reviewer #1:**



**We appreciate the insightful comments and kind remarks. We have done our best to incorporate suggested changes and provide commentary on our decision making process for this model.**

General comments:

The use of PMSG should be introduced better and it should be justified why this protocol is chosen over either estrogen injections (which is widely used to increase endometrial yield) or menstrual endometrial tissue generated via the Menstruating Mouse Model (Cousins (2014) & Greaves (2015)) (which gives a high menstrual endometrial yield and is more physiological relevant). Have you checked whether PMSG injections indeed result in increased endometrial yield compared to estrogen injections or the Menstruating Mouse Model?

**Pregnant mare serum gonadotropins (PMSG) function as FSH and LH in mice. FSH and LH act to elevate endogenous estrogen which in turn stimulates endometrial tissue proliferation. We chose to use PMSG estrogen injections for the following reasons:**

- 1. The half-life of PMSG is 40 hours whereas the half-life of 17-beta-estradiol is only 2 hours. Utilizing a single subcutaneous injection of PMSG in the donors 40 hours prior to procurement of endometrial tissue provides a sustained duration of exposure to gonadotropin stimulation which acts to stimulate endogenous estrogen. Classically, use of estrogen requires multiple injections to adequately prime the endometrium. While we did not quantify the changes seen in endometrial tissue yield in mice injected with PMSG compared to estrogen injection, we did evaluate PMSG compared to placebo in our early work. Injection of PMSG significantly increases endometrial thickness and tissue yield compared to placebo injections. This change is grossly apparent. Additionally, multiple other groups have reported on the effects of gonadotropins on endometrial proliferation (citations added to the text).**
- 2. Induction of pathways by FSH and LH leading to endogenous estrogen expression are more physiologically relevant than exogenous estrogen supplementation. Estrogen treatment may also incur off target effects on other tissues and the endometrium; prior literature has reported on alterations in progesterone receptor expression in the endometrium when estrogen is used in mice.**
- 3. The donor mice are reproductively naïve (by design) and have not yet begun estrous cycling. The endometrial tissue harvest following gonadotropin injection is timed to ensure collection of tissue before ovulation, which occurs at after 42 hours post injection in common species of mice. Ovulation would produce a high P4 environment and reduce lesion establishment.**
- 4. Other published mouse models of endometriosis have used PMSG for endometrial proliferation and shown similar success (citations added to the text).**

In text comments

Line 118: You use 22-24 day old mice to ensure maximal estrogenic response. Most strains, however, only reach sexual maturity at 6 - 8 weeks of age. Therefore, I would assume that you cannot have a maximal estrogenic response at 22-24 day due to the gonadotropin injection.

**This was an error and the sentence has been corrected to “*For timing of the endometrial tissue transplant, donor mice should be between 22-24 days old at time of gonadotropin injection to ensure they are reproductively naïve, e.g. not yet begun estrous cycling.*”**

Line 121: Why do you not synchronize the oestrus cycle of the animals with injections of oestrogen? The Whitten effect tends to have variable results and largely depends on the amount of male bedding available. Instead, three consecutive subcutaneous injections of 100 ng/100 µL oestradiol will bring all animals to the oestrus phase while the animals are still reproductively intact.

**In a larger study using both wildtype and mutant mice (publication forthcoming), we found that estrous phase of recipient was not a statistically significant factor in lesion size. Therefore, we feel that this is of lesser importance to the model, however, this is an excellent point and we have added this suggestion to the manuscript.**

Line 146: The "intima" is not the correct anatomical term when referring to the epithelial side of the endometrium. Please adjust.

**This has been corrected.**

Line 149: Do you separate the endometrium from the myometrium? Or are the tissue fragments composed of both endometrium and myometrium? Human endometriosis lesions do not display any myometrium, so implanting myometrium is not physiologically relevant.

**In creating our model, we have done both approaches (separating off the myometrium vs fragmenting together), and both approaches will create lesions. Histological examination of lesions from mice injected with tissue fragments composed of both endometrium and myometrium show only endometrial glands and stroma remaining at 3 weeks. No myometrium remains. However, we agree retaining the myometrium with the injected endometrium would give the mouse model less physiologic relevance to humans. We have added language to reflect this important point.**

Line 149: How do you perform the fragmentation? Please illustrate this in more detail.

**We have added more language describing the fragmentation, but ultimately, this was one of the reasons we felt a video manuscript was beneficial for demonstrating how to best perform the fragmentation.**

Line 173: Did you check if the take rate increases over time? (A shorter/longer time post-implantation results in less/more attached lesions)

**From our experience and from discussion with collaborators using similar models, the maximum lesion size and number occur at approximately 3 weeks post-transplant; after 3 weeks, lesions begin to regress in size. We have added language to highlight this point.**

Line 234: Is the lesion take rate the same as the disease prevalence? I would assume that the take rate refers to how many tissue fragments (average 22) have adhered to the abdominal organs (average 1.5). Therefore, in your case, the take rate is only 6.8%. The disease prevalence would rather reflect the amount of animals that displayed the disease after inoculation, which in your case is 80%.

**Thank you for this observation, we agree that our use of terminology was not accurate. This has been corrected.**

**Reviewer #2:**

**Thank you for taking the time to thoroughly review our manuscript. We have done our best to incorporate suggested changes.**

Manuscript Summary:

This goal of this methods article is to present a novel simplified murine endometriosis model that creates lesions histologically consistent with that of women with endometriosis. This model requires hormonal stimulation of donor mice and an intraperitoneal injection but does not require surgery or immunocompromised mice, often seen as limitations of other models. This is a well written coherent methods paper with very nicely constructed figures. Overall, the authors provide a novel model for studying endometriosis which may advance treatment strategies and/or diagnostic tools which are desperately needed for women suffering from the disease. I only have a few minor comments to improve the manuscript.

Major Concerns:

ESTROUS STAGE

The authors state that phase of the estrous cycle does not impact the ability to establish lesions (lines 293-294). What estrous stage was donor mouse endometrial tissue collected in?

**The donor mice are reproductively naïve (by design) and have not yet begun estrous cycling. Use of gonadotropins in this reproductively naïve mice, stimulates the exogenous estrogen production and endometrial proliferation. The endometrial tissue harvest following gonadotropin injection is timed to ensure collection of tissue before ovulation, which occurs at after 42 hours post injection in our species. Ovulation would produce a high P4 environment and reduce lesion establishment.**

The authors state that estrous phase of recipient mouse was not associated with lesion number or microscopic disease score (line 239-240). Could you elaborate on how this was determined (e.g. how many mice in each estrous stage were used to determine this)?

**Estrous phase was determined by vaginal smear cytology in recipient mice (n=40 across all experiments) at time of intraperitoneal injection (diestrus = 9, metestrus = 9, proestrus = 2, estrus = 20,). There was no difference in lesion formation based upon estrous phase in our model.**

Minor Concerns:

Title:

consider removing the word high

**We have shortened the title to “*A syngeneic murine model of endometriosis using naturally cycling mice*”.**

Figures:

Please add scale bars to figures as applicable.

**These have been added.**



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11/02/2020

**Journal of Visualized Experiments**  
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