Bioluminescent imaging in induced mouse models of endometriosis reveals differences in four model variations

Ashley Dorning¹, Priya Dhami², Kavita Panir², Chloe Hogg¹, Emma Park¹, Gregory D. Ferguson³, Diane Hargrove³, James Karras³, Andrew W. Horne¹, Erin Greaves²

1. Medical Research Council (MRC) Centre for Reproductive Health, Queen’s Medical Research Institute, The University of Edinburgh, Edinburgh, United Kingdom.
2. Centre for Early Life, Warwick Medical School, University of Warwick, Coventry, United Kingdom.
3. Ferring Research Institute, 4245 Sorrento Valley Blvd, San Diego, CA 92121

# To whom correspondence may be addressed. Email: Erin.Greaves@warwick.ac.uk ORCID 0000-0001-9165-5851

Summary statement: Different versions of syngeneic mouse models of induced endometriosis exhibit disparities in chronicity and cellular composition of lesions as well as endometriosis-associated hyperalgesia.

Abstract

Our understanding of the etiology and pathophysiology of endometriosis remains limited. Disease modelling in the field is problematic as many versions of induced mouse models of endometriosis exist. We integrated bioluminescent imaging of ‘lesions’ generated using luciferase-expressing donor mice. We compared longitudinal bioluminescence and histology of lesions, sensory behavior of mice with induced endometriosis and the impact of the GnRH antagonist Cetrorelix on lesion regression and sensory behavior. Four models of endometriosis were tested. We found that the nature of the donor uterine material was a key determinant of how chronic the lesions were as well as their cellular composition. The severity of pain-like behavior also varied across models. Whilst Cetrorelix significantly reduced lesion bioluminescence in all models, it had varying impacts on pain-like behavior.
Collectively, our results demonstrate key differences in the progression of the ‘disease’ across different mouse models of endometriosis. We propose that validation and testing in multiple models, each of which may be representative of the different subtypes/heterogeneity observed in women should become a standard approach to discovery science in the field of endometriosis.

Introduction

Endometriosis is an enigmatic, incurable condition impacting 190 million women worldwide during their reproductive years. It is associated with debilitating pelvic pain, dysmenorrhea, dyspareunia and infertility (Zondervan et al., 2020). Despite its high prevalence and devastating impact on health-related quality of life, treatment options for endometriosis remain limited and currently the main therapies to treat symptoms are surgical excision of lesions or suppression of ovarian hormones (Giudice and Kao, 2004). Recurrence is a key problem following surgical excision and both options have unwanted side effects. There is an evident unmet clinical need for new medical therapies for the treatment of endometriosis.

Endometriosis is defined by the presence of endometrial-like tissue explants usually within the pelvic cavity known as ‘lesions’. Endometriotic lesions contain endometrial-like stromal cells with or without epithelial glands, can be highly infiltrated by immune cells, vascularized and innervated (Greaves et al., 2017a). Lesions can also contain a significant fibrotic component (Vigano et al., 2018). Clinically, the histological appearance of endometriotic lesions are significant heterogeneous which can add complexity to diagnosis (Clement, 2007). Careful re-appraisals of the pathology of endometriosis highlight the variable nature of their components and suggest that typical endometriosis should contain endometroid epithelium, or stroma, or fibrosis or hemosiderin-laden macrophages. These often co-occur but not in all instances (Clement, 2007, Vigano et al., 2018). One of the most widely accepted theories for the development of endometriosis is dissemination of endometrial fragments resulting from retrograde menstruation (Sampson, 1927). However, it remains unknown why endometriosis occurs in a proportion of women when approximately 90% of women experience retrograde menstruation. Heterogeneity in disease severity and lesion subtypes (superficial peritoneal, ovarian endometrioma and deep infiltrating) suggest that endometriosis could have multiple origins and other theories are increasingly being discussed (Sourial et al., 2014).
Endometriosis only develops spontaneously in humans and some primates (MacKenzie and Casey, 1975), however mice are most frequently used as a model for discovery science and testing potential therapeutics given their economic viability and ease of manipulation. Many variations of mouse models of induced endometriosis are prevalent in the research community and the only unifying characteristic of these models is the establishment of ectopic endometrial tissue. Mouse models of induced endometriosis can be classified as autologous (auto-transplantation), heterologous (human endometrium xenografted into immunodeficient mice) or syngeneic (donor-recipient of same strain) as recently reviewed (Simitsidellis et al., 2018, Greaves et al., 2017a, Greaves et al., 2020). The most commonly utilized mouse model is the syngeneic model as this allows the use of transgenics as donor or recipients, facilitating investigation of donor / recipient derived cells and genes in the pathogenesis of endometriosis. The majority of syngeneic models are based on the transplantation of uterine material to ectopic locations within the pelvic cavity, however the nature of the donor uterine material, method and site of transplantation, as well as hormonal status of recipient mice differs between research groups. Advantages and disadvantages of each variant have previously been summarized (Greaves et al., 2017a) but this extreme heterogeneity and lack of standardization amongst endometriosis models likely contributes to diminished reproducibility and comparability across studies. Importantly, translation of preclinical endometriosis studies to clinical trials has been overwhelmingly unsuccessful and there is a distinct lack of new drugs in the pipeline (Guo, 2014, Guo and Groothuis, 2018). This very clearly highlights that we still have insufficient understanding of endometriosis aetiology and whether preclinical mouse models effectively recapitulate pathology and symptomology in women.

The impact of interventions on endometriosis lesion size and number is improved by non-invasive imaging of lesions. This facilitates the collection of data pre, during and post-drug treatment during longitudinal studies, improves experimental design and reduces the number of mice required. Some groups have used fluorescent or bioluminescent imaging to aid identification and quantification of lesion number and size (Fortin et al., 2003, Wang et al., 2014). With fluorescent models subcutaneous placement offers preferential signal detection, only a weak signal was detected from endometrium grafted intra-peritoneally (Martinez et al., 2019a). Another syngeneic model where donor uterine fragments from mice that ubiquitously express luciferase (UbC-Luc) were sutured to the peritoneal wall of wild-type mice allowed robust imaging of endometriotic lesions. The group demonstrated that bioluminescent signal correlated well with lesion size (Becker et al., 2006).
We previously developed a mouse model of induced endometriosis that aims to recapitulate the process of retrograde menstruation; endometrial breakdown is induced in a ‘menses’-like event (Cousins et al., 2014) in a donor mouse and the resulting ‘menstrual’ tissue is injected into the peritoneal cavity of ovariectomized recipient mice supplemented with estradiol. Lesions form that phenocopy features of lesions recovered from women (Greaves et al., 2014). Mice with induced endometriosis also exhibited alterations in sensory behavior and associated molecular adaptations in the nervous system (Greaves et al., 2017b). It has since been demonstrated that endometriosis lesions can be established using a minimally-invasive model, where endometrium collected from naïve mice is injected into the peritoneal cavity of intact recipients (Dodds et al., 2017). Dodds et al compared the impact of estrus stage on lesion development and investigated differences in the appearance and architecture of lesions across different strains of mice. However, there has been no direct comparison drawn between endometriosis mouse models. It remains unknown exactly how different models compare in terms of efficacy of endometrial tissue attachment and lesion longevity, cellular composition of lesions, development of endometriosis-associated hyperalgesia and most importantly, response to drug treatment. The minimally-invasive model also negates the requirement for surgical shams and has been proposed as a preferable model for the study of endometriosis-associated pain. Although no behavioral studies have been published using this model as yet, subtle changes in spinal glia were observed (Dodds et al., 2019) that would likely be precluded in surgical models.

Another similar non-surgical model used full thickness uterine tissue and the mice exhibited abdominal mechanical allodynia, spontaneous abdominal pain as well as changes in thermal selection behavior (Fattori et al., 2020).

In the current study, our aim was to integrate non-invasive bioluminescent imaging into our mouse model of induced endometriosis and to investigate how four different versions of endometriosis mouse models compare to one another. We compared lesion histology and bioluminescent signal, development of mechanical hyperalgesia in each model as well as response to the GnRH antagonist Cetrorelix.

Results

Integration of non-invasive bioluminescent monitoring of lesions in the ‘menses’ model of endometriosis. Luminescence is ubiquitous in homozygous CAG-luc-eGFP mice following subcutaneous (s.c) administration of 1.5mg luciferin. Images were acquired using a Biospace PhotonIMAGER™ (Fig.1A, top panel). Bioluminescence was also confirmed in dissected uteri
To allow non-invasive bioluminescent detection of endometriosis ‘lesions’ in our established model, endometrial tissue from donor CAG-luc-eGFP mice manipulated to undergo a ‘menses’-like event was collected as previously described (Cousins et al., 2014, Greaves et al., 2014). This tissue was injected into the peritoneal cavity of wild-type (non-luminescent) recipient mice (Greaves et al., 2014) (Fig.1B). Twenty-one days after intra-peritoneal (i.p) injection of ‘menses’-like endometrial tissue, bioluminescent endometriotic lesions could be detected in the peritoneal cavity of recipient mice following s.c administration of luciferin (Fig.1C). When administration routes of luciferin were compared (7 days post injection of tissue) it was noted that i.p injection in some cases produced a greater bioluminescent signal compared to smaller localized foci following s.c injection in the same mouse (Fig.1D). In these animals, unattached material was found in the peritoneal cavity on dissection. This indicates that s.c administration of luciferin produces bioluminescent signal from attached, vascularized implants of endometrial tissue only, and not unestablished ‘floating’ endometrial tissue. Following dissection of lesions at the end of the experiment (Fig.1E) we validated donor origin by confirming expression of eGFP (Fig.1F) by endogenous fluorescence imaging and expression of luciferase (Fig.1G) by immunofluorescence. Lesions exhibited expected histology upon H&E stain comprising of stromal with or without glandular components (Fig.1H). Thus, we have established bioluminescent non-invasive imaging of lesions in our ‘menses’ mouse model of induced endometriosis that enables clear localization of vascularized lesions. Moreover, donor origin of lesions can be verified using endogenous fluorescence or immunofluorescence.

Bioluminescent monitoring in different models reveals variations in lesion luminescence and a gradual resolution of lesions over time. In our established ‘menses’ model of induced endometriosis, recipient mice are ovariectomized and receive estradiol supplementation (Greaves et al., 2014) (hereafter referred to as ‘DO’ - decidualized (‘menses-like’) endometrium into ovariectomized recipients; Fig.1B). The model negates the ability to assess impacts on fertility or therapies that target ovarian signaling, and introduces further complexities in experimental design by requiring the inclusion of surgical sham controls that have also had an ovariectomy performed. Thus, we chose to adapt the model to use intact recipient mice (referred to as ‘DI’ - decidualized endometrium into intact recipients; Fig.2A). Additionally, several other variations of endometriosis mouse models exist that vary in the nature of uterine material introduced into the peritoneal cavity. Thus, we compared the two variations of our menses model of endometriosis to a minimally invasive model (Dodds et al., 2017) where naïve donor endometrium from cycling mice is injected i.p into intact
recipients (hereafter referred to as ‘NI’ - naïve endometrium into intact recipients; Fig.2B) as well as full thickness uterine fragments (including myometrium) into recipient mice (‘MI’ model [full thickness including myometrium into intact recipients; Fig.2B] in our study. We initially aimed to ascertain any key differences in lesion bioluminescence or longevity originating from the type of donor tissue used to establish endometriotic lesions. At day (d)21 post-tissue inoculation, the bioluminescent signal intensity was significantly greater in the MI model compared to the NI (p<0.001), DI (p<0.01) and DO models (p<0.05; Fig.2C). To assess differences in lesion longevity across models, we recorded the number of mice with at least one bioluminescent lesion (when luciferin was administered s.c) at d10, 21, 31 and 42 post tissue injection. At d7 post tissue injection 90% of mice in the DO and DI group had lesions, 96.6% of mice had lesions in the NI group and 100% had lesions in the MI group. At d21, 60% of DO mice had bioluminescent lesions, whilst 80% of DI mice, 66% of NI mice and 90% of MI had lesions (Fig.2D). We also repeated this analysis at d42 post tissue injection and identified that 40% of DO mice still had lesions, 50% of DI mice and only 31% of NI mice still had bioluminescent lesions. In MI mice, 71% still had lesions (Fig.2D). Longitudinal imaging of mice indicated that in the DO and DI models, lesions show a progressive decline in size, and between 50-60% of lesions eventual resolve by 6 weeks post-tissue injection (Fig.2E-H). However, a greater degree of variability in lesion signal intensity is evident in the NI and MI models.

Observation of individual mice over time also revealed that in these alternative models some lesions exhibit spontaneous resolution whilst others progress in size; we have shown images of two mice from the NI group (Fig.2G) that clearly illustrates the observed variability within this group; the mouse on the left shows an increase in bioluminescence from d10 to 42 (with a newly detectable bioluminescent foci representative of a new lesion at d42) whereas the mouse on the right has 5 localized lesions 10 days after tissue injection that resolve by d21. We observed similar variability in the MI group. In the two representative images presented in Fig.2H the lesion detected in the mouse on the left exhibits a gradual decline in size, whilst the two lesions detected in the mouse on the right exhibit an increase in size from d10 to d42. In the NI group we found no difference in signal intensity of lesions in recipient mice across the estrus cycle (Fig.S1A) suggesting that one source of variation could be due to estrus stage of the donor or the recipient at the time of tissue injection (as previously observed by Dodds et al 2017). When we separated out the animals based on estrus stage we found that 100% of mice that received estrus donor endometrium had lesions at d21 (Fig.S1B). This suggests that lesion establishment /
longevity could be greater when estrus phase donor endometrium is injected, however the differences are negligible thus donor endometrial / uterine material from different stages was pooled and divided between recipients going forward in order to reduce any variation. Estrus stage of the recipient at tissue transfer appeared to have no impact on number of lesions at d21 (Fig.S1B).

**Glandular content varies but fibrosis is a consistent feature of lesions collected from different mouse models of endometriosis.** All biopsies collected from the peritoneal cavity of mice with induced endometriosis (d42 after tissue injection) were stained with hematoxylin and eosin (Fig.3A-D). At this point we positively selected samples that exhibited expected lesion architecture (endometrial-like stromal cells with or without epithelial cells). From the DO model we recovered only 3 lesions from 10 mice (30% in contrast to 40% detectable lesions via imaging). We had 4 additional lesions from this model and time-point (from previous experiments) that we could include in the histological analysis in order to increase the sample size to n=7 lesions. We recovered 5 lesions from 10 mice in the DI model in line with the 50% of mice with detectable lesions via imaging. From the NI model we recovered only 5 lesions from 29 mice at d42 post tissue injection (17.24% in contrast to 31% detectable via imaging). From the MI model we recovered 23 lesions from 20 mice (71% of mice had detectable lesions at imaging; we recovered lesions from 12 out of 14 mice with positive signal as well as a further 3 mice that did not exhibit signal. Thus, the total recovery was 75%). All positively identified lesions were immuno-positive for vimentin (Fig.3E-H), a mesenchyme marker, indicating the presence of endometrial-like stromal cells in lesions collected from all 4 models of endometriosis. We also performed immunodetection for cytokeratin to assess presence of endometrial-like glandular epithelium (Fig.3I-L). Initially, we quantified the percentage of lesions that contained cytokeratin-positive glandular structures (see representative images Fig.3I-L); in the DO group 40% of lesions had glands, 20% in the DI group, 80% in the NI group and 92% in the MI group (Fig.4A). We also quantified the number of lesions that had cytokeratin-positive cells but without evidence of a glandular lumen. In the DO and DI group 70% and 60% of lesions (respectively) had cells that were immuno-positive for cytokeratin whilst 100% of lesions from the NI and MI group had cells that were cytokeratin positive. (Fig.4A). To observe the deposition of collagen as a measure of fibrosis in lesions we performed a Picrosirius stain (Fig.3M-P). All lesions exhibited some areas of dark pink stain (collagen) and when the area of fibrosis is each lesion was quantified, we identified no significant difference across the different models (Fig.4B). This indicates that fibrosis is a consistent and equal feature in each endometriosis
model at the time-point analyzed. Finally, we performed immuno-detection for alpha-smooth muscle actin (α-sma; Fig.3Q-T), which can be a marker of collagen producing fibroblasts. In the DO and DI models 86% and 100% of lesions (respectively) were immuno-positive for α-sma. In the NI and MI models 80% and 100% of lesions were immuno-positive for α-sma (Fig.4C). These results further support the indication that fibrosis is consistent across the models. We also recorded the location from which lesions were recovered (Fig.4D). In the DO model, lesions were equally distributed between the parietal peritoneal lining and organ-associated fat and mesentery. In the DI model 20% of lesions were also recovered from other locations such as on the bladder and outer wall of the uterus. In the NI model 67% of lesions were recovered from the peritoneal wall, 29% from organ associated fat and only 4% from other locations. In the MI model 46% of lesions were recovered from the peritoneum, whereas the remaining lesions were evenly distributed between fat and other locations. There was no significant difference in lesion size across the different models (Fig.4E).

Changes in sensory behavior are evident in different mouse models of endometriosis. We have previously demonstrated that the ‘menses’ model of endometriosis (DO) exhibits mechanical hyperalgesia when von Frey filaments are applied to the abdomen and the hind-paw (Greaves et al., 2017b). In the current study, when von Frey filaments were applied to the abdomen of mice in the DO model we did not detect a significant difference in abdominal retraction between endometriosis and sham (OVX+EV+PBS i.p) mice (Fig.5A), contrary to our previous observation. We postulate that this may be due to differences in mouse strain which in this study was FVB/N but in our prior study was C57Bl/6. In the DI and NI models, mice with endometriosis exhibited significantly lower abdominal retraction thresholds compared to sham animals (p<0.05 and p<0.01 respectively; Fig.5B). Abdominal retraction threshold was lower in sham-ovx compared to sham-intact although this did not reach statistical significance (Fig.S2A), but along with results shown in (Fig.5A and C) illustrates that sham-ovx mice exhibit changes in sensory behavior associated with previous surgical procedures and indicates that intact models are preferable for assessment of pain-related behaviors. Endometriosis mice in the DO group did exhibit significantly reduced paw withdrawal thresholds compared to sham-ovx (p<0.001; Fig.5C) indicating that referred hyperalgesia consistent with central maladaptation can be detected in this group and is consistent with our previous studies(Greaves et al., 2017b). Paw withdrawal threshold was significantly lower in endometriosis mice from the DI group compared to sham-intact mice.
Mice from the NI and MI groups did not exhibit reduced paw withdrawal thresholds compared to sham-intact mice.

**GnRH antagonism attenuates lesion growth in all intact models, but only fully rescues pain response in the NI model.** Next, we tested the performance of the 3 intact models in response to a therapy known to attenuate endometriosis symptoms in women. We induced endometriosis in mice and at d7 post-tissue injection we imaged lesions to obtain a ‘pretreatment’ baseline. Mice were then randomized into two groups to receive subcutaneous injection of either vehicle (H2O) or GnRH antagonist (Cetrorelix; 20mg/kg) every 48h for 2 weeks (Fig.6A). Administration of Cetrorelix resulted in a significant decrease in ovarian weight (Fig.S2A). Although Cetrorelix reduced lesion signal intensity in the DI model (Fig.6B-C; p<0.001) it did not have a significant impact on pain response (Fig.6D-E) when von Freys were applied to the abdomen or hindpaw. In the NI model Cetrorelix attenuated lesion signal intensity (Fig.6F-G) and pain response when filaments were applied to both the abdomen (Fig.6H; p<0.001) and hindpaw (Fig.6I; p<0.05). Cetrorelix also reduced lesion signal intensity in the MI model (Fig.6J-K; p<0.01). Local pain response was attenuated when von Frey filaments were applied to the abdomen (Fig.6L; p<0.01), but there was no difference in referred pain hyperalgesia when von Frey filaments were applied to the hindpaw (Fig.6M).

**Discussion**

In our current study, we have assessed the utility of bioluminescent imaging in our existing mouse model to allow us to make repeated measurements of lesion number and size longitudinally. We then compared our existing model to three different variations of mouse models of induced endometriosis in order to compare differences in bioluminescent signal, lesion histology, pain-related behavior and response to a cogent therapy: Cetrorelix (GnRH antagonist). Our main findings were five-fold: 1) lesion signal intensity was significantly greater in the MI model (full thickness uterine fragments in intact mice), this also translated to a greater number of lesions per mouse. 2) A gradual resolution of lesions was evident in all models, however in the NI (naïve endometrium into intact mice) and MI models some lesions exhibited evidence of progression in size and occasional development of additional bioluminescent foci suggesting the formation of new lesions. 3) The presence of glandular epithelium in lesions varied across models, with more detected in the NI and MI models, whereas the presence of fibrosis was consistent. 4) The degree of changes in
sensory behavior varied across models, the most significant changes were evident in the DI model (‘menses-like’ endometrium into intact recipients), where robust sensitization was observed at the abdomen and hindpaw. 5) Administration of Cetrorelix decreased lesion bioluminescent signal intensity in all models, but its ability to reduce changes in sensory behavior varied between models. Taken together, these data demonstrate evident differences between mouse models that need to be carefully considered during the design of in vivo experiments.

The finding that many lesions gradually decrease in size over time and approximately half spontaneously resolve by 6 weeks, whereas a small proportion of mice in the NI and MI models exhibit progression of bioluminescent foci was intriguing since this is known also known to occur in non-human primates and women. Resolution was evident when recipients were ovariectomized and supplemented with estradiol or left with their ovaries intact, suggesting this phenomenon occurs regardless of the hormonal status of mice. Endometriotic lesions are known to evolve during active disease, as evidenced by the changing colour of lesions over time. Baboons with experimentally induced endometriosis that were subject to multiple laparoscopies demonstrated a large proportion of red active lesions shortly after disease induction which changed colour to blue, chocolate, white and mixed pigmentation as the disease progressed (Harirchian et al., 2012, Hastings and Fazleabas, 2006). Spontaneous resolution of lesions was also evident in the Baboon model, with only 41% of the initial lesions identifiable at the end of the study (15 months post inoculation). However, at 12 months after tissue inoculation 51% of lesions present were newly detected lesions, indicating that in baboons endometriosis can be progressive (Harirchian et al., 2012). Observational studies in women also suggest that lesions are dynamic. Second-look laparoscopy studies reported by Sutton et al indicated that of the patient population studied 29% showed some regression of disease, whereas the remaining majority progressed or remained stable (Sutton et al., 1997).

Thus, our modelling experiments in mice do mirror the fluctuating nature of ectopic tissue to a certain extent, although increased levels of resolution and lower levels of progression are evident in mice with induced endometriosis. This finding can be extrapolated to a new concept: that instead of modelling ‘endometriosis’, in a large proportion of mice we are in fact modelling a ‘healthy’ response to refluxed endometrial tissue. This can be exploited as it allows rationale to be developed on processes that might be defective in women with endometriosis. It remains unknown what is different about the lesions that progress / are maintained long-term and future work should draw comparisons
between resolving vs non-resolving mice. Recently, we identified a ‘protective’ population of monocyte-derived large peritoneal macrophages (LpM) in our menses mouse model of endometriosis that limit the development of lesions (Hogg et al., 2021). Using gain and loss of function experiments we demonstrated that depletion of this population increases development of lesions, whilst re-programming the peritoneal niche such that embryo-derived LpM are ablated, and the niche is repopulated afresh with monocyte-derived LpM leads to decreased lesion development. These data support the idea of immune dysfunction in women with endometriosis (Ahn et al., 2015) which requires further exploration. The resolving vs non-resolving platform can also be exploited to further define this peritoneal immune environment in response to continued, progressive or resolved endometriosis.

In the NI and MI mouse models we occasionally visualized progressive lesions (either new foci or growth of lesions). This suggests that the ‘menses-like’ material used to inoculate recipient mice in the DO and DI model may not contain the cell-types required to establish chronic or progressive endometriosis. The cellular origins of endometriosis have yet to be fully elucidated (Filby et al., 2020). However, it has been postulated that endometrial stem / progenitor cells are the cells of origin of endometriosis lesions (Gargett, 2007, Cousins et al., 2018). Previous studies in a mouse model of endometrial breakdown and repair have identified that only 0.14% of decidual cells are label-retaining cells prior to breakdown (Kaitu'u-Lino et al., 2012), suggesting that very few putative stromal stem-like cells are transferred into recipient mice in the DO and DI models. Evaluation of menstrual blood collected from women with and without endometriosis suggests that mesenchymal stem-like cells and epithelial progenitors are present in menstrual effluent, with a trend towards increased progenitors in women with endometriosis(Masuda et al., 2021). This highlights that there are disparities in progenitor populations shed in women vs mice induced to menstruate. A two-stem / progenitor cell hypothesis has also been proposed whereby both stromal and epithelial progenitor cells are required to form typical endometriosis lesions with both stromal and epithelial compartments (Wang et al., 2020). Based on the results presented in the current study, we postulate that a higher proportion of both stromal and epithelial progenitors are present in the donor material in the NI and MI models as lesions recovered from these models exhibit higher levels of histologically confirmed glandular epithelium and these models show some progression whereas the other models do not. Interestingly, we found that a greater number of mice had lesions at d21 when estrus stage endometrium was introduced into the peritoneal cavity. It has recently been shown that the number of stem-like cells fluctuates across the estrus cycle in...
mice and that an elevated number can be detected in the estrus stage (Singh and Bhartiya, 2021), thus adding further evidence for a role for stem cells. However, despite pooling tissue from different estrus stages to reduce variability we still only observed progression in a few mice. Thus, at present we are unable to conclude exactly what determines chronic lesions or those prone to progression or resolution. In the models that use donor ‘menses’ endometrium (DO and DI), decidualization is artificially induced (Greaves et al., 2014). However, women with endometriosis reportedly exhibit impaired decidualization with stromal fibroblasts present in menstrual effluent also demonstrating an impaired ability to decidualize when cultured in vitro (Warren et al., 2018). One possible explanation for increased resolution in the DO and DI models is that decidualized stromal fibroblasts may exhibit a lower propensity to form chronic lesions, although this requires further investigation.

It is also important to highlight that the variability of lesions containing glandular structures is consistent with clinical findings that not all lesions possess evident epithelium. These lesions are often diagnosed as ‘stromal endometriosis’ (Clement, 2007). The variability in the occurrence of fundamental lesion components across the models reflects the heterogeneity of lesions found in women and may have key implications for response to therapy. For example, ‘stromal endometriosis’ may respond differently to ‘traditional’ lesions that possess epithelium. To this end it has been proposed that treatment resistance in endometriosis could be associated with the evident fibrosis present within lesions and this component (Groothuis and Guo, 2018) may require alternative treatment possibly in combination with hormone therapy.

It is striking that despite controlling for the amount of tissue transferred per animal, the establishment, longevity and signal intensity of lesions in the MI model far out-performs the other models. However, despite this model exhibiting traditional lesion histology (robust evidence of glandular structures in all lesions) the impact on pain response is minimal compared to the DI model. This has led us to speculate that perhaps the different models could represent different subtypes of lesions. The DO and DI models which use ‘menses-like’ endometrium to initiate lesions may represent an inflammatory subtype such as red active lesions. The finding that sensory behavior modifications were more pronounced in the DI model and that Cetrorelix was not able to rescue these changes further substantiates this idea and that this model could be compared to endometriosis that is refractory to hormonal treatment in women. However, it has been reported that red peritoneal lesions contain proliferative glandular epithelium (Nisolle and Donnez, 1997), whereas limited number of
lesions from the DO and DI models contain cytokeratin positive gland structures. One limitation of the current study is that we only used a measure of evoked mechanical hyperalgesia to assess sensory behavior and should we have included further assessments, the MI model may have exhibited a more severe sensory phenotype. To this end, the non-surgical model published by Fattori et al (using full thickness uterus as donor material) exhibited abdominal mechanical pain as well as spontaneous pain-related behavior. The mice did not exhibit thermal hyperalgesia or any differences in dynamic weight bearing (Fattori et al., 2020). Recently, a mouse model of deep infiltrating endometriosis has been established, where mice with induced endometriosis were infused with the neurotransmitters substance P and calcitonin gene related peptide via osmotic pumps. The lesions recovered from mice exhibited hallmarks of deep infiltrating lesions including presence of endometrial epithelial and stromal cells, abundance of fibromuscular content, encapsulation in surrounding tissues or organs and extensive fibrosis. The mice also exhibited thermal hyperalgesia in response to the hotplate test (Yan et al., 2019), but spontaneous behaviour was not recorded. Future studies should aim to incorporate a wide range of behavioural tests such that parallels between studies can be drawn more easily.

Cetrorelix (GnRH antagonist) is a preferable hormone therapy for endometriosis compared to previously used GnRH agonists because fewer side-effects occur and no estradiol add-back is required (Finas et al., 2006). A study including sequential laparoscopic and clinical evaluation before and after Cetrorelix therapy in women with endometriosis revealed a reduction in disease stage for women with minor endometriosis (Küpker et al., 2002). Regression occurred in 60% of cases, however in women with severe (ASRM (American Society for Reproductive Medicine) stage IV) endometriosis Cetrorelix had no impact (Küpker et al., 2002). In the current manuscript all three intact mouse models exhibited a reduction in lesion bioluminescent signal intensity and a visible reduction in bioluminescent foci following treatment with Cetrorelix. Despite this desirable effect on lesions, attenuation of mechanical hyperalgesia was only observed in the NI and DI models, highlighting disparities in response to this established therapy for treatment of endometriosis-associated pain.

We have proposed a blueprint that summarizes the features and utilities of the different mouse models analyzed in this study (Fig.7). The DO and DI models that use ‘menses-like’ endometrium for the formation of lesions aim to recapitulate the physiological process of retrograde menstruation, however they perform less well in displaying the traditional lesion architecture of well-developed glandular structures. The DI and NI
demonstrate the most significant changes in sensory behavior whilst only the NI and MI exhibit a progressive phenotype in some mice. We have not observed ovarian or deep infiltrating lesions in any of the models. However, recent advances have report a genetically engineered model that mimics the natural spread of invasive endometrium including formation of lesions on the ovary (Wilson et al., 2020). Taken with our findings that include a predicted inflammatory phenotype (the ‘menses’ model) that might be refractory to hormonal suppression and the above mentioned model of deep infiltrating endometriosis (Yan et al., 2019) the field now has a toolbox of models that may be compared to different subtypes of endometriosis and could be used in combination for testing of promising innovative treatments for endometriosis.

Our studies highlights variability in the performance, and possibly underlying ‘disease’ mechanisms of different mouse models model of induced endometriosis. Currently, we do not know enough about the pathophysiology and ontogeny of endometriosis to be able to suggest a standardized model. Rather, the evident variability in models might also reflect different subtypes and heterogeneity of response in women. Thus, we suggest that there is a requirement for preclinical testing in multiple ‘models’ in order to ascertain how robust a potential treatment might be in targeting endometriosis and we call for international collaboration in testing of potential therapies across Institutes and models in order to generate robust preclinical data that may have greater success in clinical studies.

In summary, we have presented data indicating that not all mouse models of induced endometriosis are equal and that the nature of the ‘donor’ uterine material is an important indicator for the presence of epithelial glandular structures and long-term maintenance / progression of induced lesions. Importantly, we have demonstrated that using ‘menses-like’ endometrium produces transient lesions that exhibit a high degree of resolution by 6 weeks, although the hyperalgesia that develops in this group is more severe compared to the other models and is also resistant to GnRH treatment (although lesion bioluminescence is reduced). We propose that the differences evident in each of the models reflect the heterogeneity / subgroups observed in women with endometriosis and may reflect discrete differences in disease mechanisms and etiology. These findings are vital for the field of discovery science and preclinical testing of potential therapeutics in mouse models of induced endometriosis and support the concept of testing therapies in multiple models and may help in identifying subgroups that respond / do not respond to particular therapies.
Methods

Animals and reagents. FVB-Tg(CAG-luc,-GFP)L2G85Chco/J; stock number 008450|L2G85) were purchased from The Jackson Laboratory (ME, USA), bred and maintained in specific pathogen-free facilities at the University of Edinburgh and the University of Warwick, UK). A breeding stock of wild-type FVB mice was maintained to produce experimental cohorts of recipient mice, or bought from Charles River (UK). All experiments were permitted under license by the UK Home Office and were approved by the University of Edinburgh and University of Warwick Animal Welfare and Ethical Review Body. Mice had access to food and water *ad libitum*. Ambient temperature and humidity were 21°C and 50% respectively. To visualize bioluminescent endometriosis lesions, the substrate D-luciferin (1.5mg/100ul in PBS; Sigma-Aldrich, Dorset, UK) was injected subcutaneously (s.c) prior to imaging. Female mice between the age of 8 and 12 weeks were used for the experiments. Mice with induced endometriosis were administered Cetrorelix acetate 20mg/kg in dH2O s.c (Sigma-Aldrich), every 48 hours. Dose selected based on previous *in vivo* studies (Danforth et al., 2005, Otto et al., 2012). For the Cetrorelix experiments mice were randomly allocated to either vehicle or drug group and the investigator performing von Frey testing was blinded to experimental group.

Endometriosis modeling in mice

*Menses* mouse model of endometriosis. Endometriosis was induced in mice as previously described (Greaves et al., 2014). Briefly, donor CAG-luc-GFP mice were ovariectomized and exposed to a hormone schedule of estradiol followed by estradiol + progesterone and decidualization stimulus. Progesterone withdrawal was then initiated to induce endometrial breakdown and shedding akin to menstruation (Cousins et al., 2016). The decidualized endometrial mass was scraped away from the underlying myometrium, re-suspended in saline, passed through an 18-gauge needle once and injected into the peritoneal cavity of wild-type FVB/N mice (bred in house or bought commercially). Recipient mice were subject to ovariectomy (ovx) and estradiol supplementation (500ng estradiol valerate twice weekly). We refer to these mice as ‘DO’ (decidualized endometrium into ovx recipients; n=10). A group of sham animals were included for behavioral assessment (n=5), these animals had undergone ovx and received the same estradiol supplementation as endometriosis mice. Shams were subject to an intra-peritoneal injection of saline instead of endometrial tissue. We also used ‘menses’ endometrium to induce endometriosis in intact mice, referred to as ‘DI’ (decidualized into intact, n=10). Intact shams for this group were injected with saline...
instead of tissue (n=7). The amount of endometrial tissue used to inoculate the peritoneal cavity in each model was standardized to approx. 40 mg.

**Minimally invasive mouse models of endometriosis.** We also used 2 variations of the minimally invasive mouse model of endometriosis (Dodds et al., 2017). In the first version endometrium was dissected away from the myometrium using sharp dissection and fragments (approx. 40 mg tissue) were re-suspended in saline, passed through an 18g needle once then injected into intact recipients (n=29; ‘naïve into intact’ referred to as ‘NI’). Estrus stage was determined in donors and recipients by vaginal smear and retrospective cytological analysis (McLean et al., 2012). In the second version whole uterus was sliced into small fragments and re-suspended in saline (approx. 40 mg) and passed through an 18g needle prior to intra-peritoneal injection into intact recipients (‘endometrium and myometrium into intact’ referred to as ‘MI’; n=20). Sham animals (n=7) were included for behavioral assessments. These animals received intra-peritoneal injection of saline instead of endometrial tissue.

**In vivo optical imaging of luciferase activity and bioluminescence quantification.** Following anesthesia with isofluorane mice were injected subcutaneously with 1.5 mg luciferin potassium salt. Following a five minute incubation to allow the luciferin to be circulated and a plateau of bioluminescent activity to be reached, mice were imaged using a PhotonIOMAGER™ (BIOSPACE LAB, Paris, France). Luminescence level was measured in regions of interest (ROIs) corresponding to the pelvis, abdomen and torso combined. We used the same ROI across experiments and captured bioluminescent activity on the front and then the back of each mouse for 7 minutes. A reading of photons/second/cm²/sr was calculated using the M3 Vision software (BIOSPACE LAB), background reading was subtracted and then the reading from the front and the back of the mouse averaged.

**Behavioral assessment.** Mechanical hyperalgesia in mice with induced endometriosis was measured using calibrated Semmes-Weinstein von Frey filaments (Stoelting, Wood Vale, IL), according to the manufacturer’s instructions and as previously described (Greaves et al., 2017b). Briefly, mice were allowed to acclimatize to the apparatus and then filaments were applied perpendicular to the abdomen or plantar surface of the hind-paw in ascending order. The weight of the filament that caused a withdrawal reflex in 50% of applications was recorded. The investigator performing the measurements was blinded to experimental
group. Behavior assessment was performed on days 40, 41 and 42 post tissue injection and the mean reading across the three days plotted for each mouse. For the Cetrorelix studies, readings were taken on day 19, 20 and 21 and the mean recording plotted for each mouse.

Histology and immunodetection. Following fixation, mouse lesions were processed into 5μM sections on slides and stained using H&E; only lesions containing identifiable stroma +/- glandular epithelium were used for further analysis. For analysis of collagen deposition by Picro Sirius Red (PSR) stain sections were de-waxed in xylene and rehydrated. Sections were incubated with Picro Sirius Red dye for 2 hours, washed, dehydrated and cleared in xylene prior to mounting. Single colour immunohistochemical analysis was performed according to standard protocols and as previously described (Greaves et al., 2014). In brief, citrate antigen retrieval and blocking of endogenous peroxidase activity in 3% H$_2$O$_2$ performed. Non-specific epitopes were blocked using a species-specific serum blocking solution (1:4 serum in Tris-buffered saline + 5% bovine serum albumin). Sections were incubated with appropriate primary antibody (see Table 1 (Chung et al., 2019, Yuri et al., 2015, Martinez et al., 2019b)) overnight at 4°C in a humidified chamber. Sections were then incubated with species-specific peroxidase conjugated secondary antibody (Immpress, Vector labs) for 30 minutes at room temperature. Antibody staining was visualized by incubating sections with 3,3-diaminobezidine (DAB) for 10 minutes, followed by dehydration, clearing in xylene and mounting. Imaging was performed using a Zeiss Z1 Imager microscope or EVOS cell imaging system (ThermoFisher Scientific). All antibodies were previously validated and specificity of the secondary antibody was confirmed in our experiments using negative controls (omission of primary antibody).

Quantification of Picro Sirius stain. Analysis of collagen deposition was achieved using ImageJ. The image scale was set and the image cropped and background removed. The ‘select’ tool was used to draw round the lesion outline and measure the area of the lesion. The image was then converted to ‘RGB’ channel stack. The green channel was used to set the threshold so that only the area stained red with PSR was quantified. The area of collagen deposition (red stain) was then calculated as a percentage of the total lesion area.

Statistical analysis. Mechanical hyperalgesia measurements generate the most variation, thus sample sizes were determined on our previous experiments that detected a statistically significant difference in withdrawal threshold between sham and endometriosis...
mice (Greaves et al., 2017b). Normality of data was assessed using Shapiro-Wilk and Kolmogorov-Smirnov tests. Data did not pass normality tests and so non-parametric analyses were performed. Statistical analysis was performed using a Kruskall-Wallis with Dunn’s multiple comparison test when comparing more than two groups, and a Mann-Whitney test for comparing two groups only.

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**Competing interests.** No competing interests declared.

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**References**


Figures

**Figure 1:** Establishment of non-invasive bioluminescent imaging in the ‘menses’ model of induced endometriosis.

A. Left to right: Cag-luc-eGFP mouse injected subcutaneously (1.5mg in 100µl volume) with luciferin, Cag-luc-eGFP mouse with no injection, wild-type FVB/N mouse injected s.c with luciferin. Whole body imaging (top panels) and dissected uteri from corresponding mice (bottom panels).

B. Schematic representation of the ‘menses’ mouse model of endometriosis (also referred to elsewhere in the manuscript for brevity as ‘DO’; decidualized tissue into ovariectomized recipient). ‘Menses’ endometrium from Cag-luc-eGFP mice was injected i.p into ovariectomized (with add-back estradiol) wild-type FVB/N recipient mice. Whole body bioluminescent imaging was performed 21 days post tissue injection. C. Bioluminescent focal lesions were localized to the abdominal region of the mice.

D. Illustrates the difference between i.p (left panel) and s.c (right panel) administration of luciferin at day 7 post tissue inoculation (in the same mouse) and indicates that s.c administration can differentiate between attached explants and unattached, floating endometrial tissue.
E. At dissection lesions were observed attached to the abdominal wall, fat and organs.
F. PFA (4%) fixed lesion tissue was stained for luciferase (red), with nuclei stained with DAPI (blue).
G. Endogenous GFP (green) expression by lesions was visualized by live fluorescence microscopy prior to fixation. Surrounding GFP negative tissue can be observed (phase white).
H. Representative H&E image of a lesion with stromal cells +/- glandular epithelium and presence of hemosiderin.
Figure 2: Comparison of lesion luminescence and longevity across different models of endometriosis.

A. Schematic representation of the adapted ‘menses’ intact mouse model of endometriosis (DI). ‘Menses’-like endometrium from Cag-luc-eGFP was injected i.p into intact wild-type FVB/N recipient mice.

B. Schematic depicting the minimally invasive mouse models of endometriosis (NI and MI). Naïve endometrium from intact Cag-luc-eGFP was injected i.p into wild-type FVB/N recipient mice or full thickness uterus (including myometrium) was injected into intact wild-type FVB/N recipient mice.

C. At 21 days post tissue injection the MI model exhibited significantly higher bioluminescent signal compared to all other models (DO n=9, DI n=10, NI n=29, MI n=20). Sample sizes are representative of mice (not lesions) and were achieved by performing experiments 2-4 times.
D. The percentage of mice with focal bioluminescent lesions was quantified at 7, 21 and 42 days post tissue injection. This showed a progressive decline in the number of mice that had detectable lesions in all 4 models of induced endometriosis.

E-H. Representative longitudinal images of individual mice from day 10-42 post tissue injection. (E) Representative images of an individual mouse from the original menses model (DO), (F) the adapted menses model (DI), (G-H) two individual mice from the minimally invasive models (G: NI and H: MI). In (G), two mice are presented to illustrate the variation across individual recipients in these two groups. The mouse on the left has a large lesion at day 10, which is maintained at day 42. The mouse on the right has 4 focal lesions at day 10 that are spontaneously resolved by day 42. In (H), two mice are presented. The mouse on the left has one lesion at day 10 which has resolved at day 42 whereas the mouse on the right has 2 lesions on day 10 which progressively increase in size.
Figure 3: Identification of endometriosis ‘hallmarks’ in lesions derived from different mouse models of endometriosis.

A-D. Representative hematoxylin and eosin stains from each model. Scale bar=200μm.

E-H. Immunodetection of Vimentin to visualise stromal fibroblasts. Scale bar=50μm. Inset on D is a negative control section of whole uterus (primary antibody omitted).

I-L. Immunodetection of Cytokeratin to visualise epithelial cells. Scale bar=50μm. Inset on G is a negative control section of gut (primary antibody omitted).

M-P. Picro Sirius stain to identify areas of collagen deposition as a marker of fibrosis. Scale bar=200μm. Red stain indicates presence of collagen fibres.

Q-T. Immunodetection of α-smooth muscle actin, a marker of myofibroblasts. Scale bar=50μm. Inset on M is negative control section of gut (primary antibody omitted).
Figure 4: Quantification of histological structures in lesions recovered at d42 post tissue injection.

A. Percentage of lesions that were immuno-positive for Cytokeratin (irrespective of glandular structure; +ve stain) and the percentage of lesions that exhibited Cytokeratin positive glandular structure (DO n=7 lesions, DI n=5 lesions and NI n=5 lesions, MI n=23 lesions).

B. Area of collagen deposition (as a measure of fibrosis) in lesions from the different models.

C. Percentage of lesions that were immuno-positive for α-smooth muscle actin (α-sma).

D. Location of lesions recovered from the different models.

E. Area of lesions measured using ImageJ.
Figure 5: Changes in sensory behavior are evident in different mouse models of endometriosis.

A-B. Mechanical hyperalgesia measured using von Frey filaments applied to the abdomen. (Sham Ovx n=10, DO n=17, Sham intact n=7, DI n=10, NI n=29).

C-D. Mechanical hyperalgesia measured using von Frey filaments applied to the hind-paw. Values plotted are an average of measurements taken over three days from day 40, 41 and 42. Data are presented as means with 95% confidence intervals. Statistical significance was determined using Mann-Whitney or Kruskal-Wallis tests. *:p<0.05, **:p<0.01, ***:p<0.001.
Figure 6: Response of intact endometriosis models to Cetrorelix.

A. Schematic showing the treatment and imaging schedule of DI, NI and MI mice.

B. Percentage change in lesion bioluminescent signal calculated from vehicle (H2O) and Cetrorelix treated mice with endometriosis (DI group). % change = (21d signal- 7d signal)/d7 signal*100. Vehicle n=22, Cetrorelix n=17.

C. Representative images from d7 (pre-drug) and d21 (end of experiment, DI group).

D. Mechanical hyperalgesia measured using von Frey filaments applied to the abdomen.

E. Mechanical hyperalgesia measured using von Frey filaments applied to the hind-paw.

F. Impact of Cetrorelix treatment on lesion bioluminescent signal intensity (p<0.05) in the NI group. Vehicle n=13, Cetrorelix n=10.

G. Representative images from d7 (pre-drug) and d21 (end of experiment, NI group).

H-I. Impact of Cetrorelix on mechanical hyperalgesia at the abdomen (H) and hind-paw (I) in the NI model.

J. Impact of Cetrorelix on lesion signal intensity in the MI group. Vehicle n=17, Cetrorelix n=17.

K. Representative images from d7 and d21 (MI group).

L-M. Impact of Cetrorelix on mechanical hyperalgesia when von Frey filaments were applied to abdomen (L) and hind-paw (M).
Fig. 7. Blueprint of models compared in the study. We have used a traffic light scoring system to denote whether a model performs well (green), partially (orange) or not at all (red) in modeling a particular feature or outcome. Note: we have scored the intact models as green for measuring fertility outcomes because they have the ability to be used in this context, however we have not yet demonstrated any differences in pregnancy outcome in the models.
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Fig. S1. Impact of estrus stage on lesion signal intensity and longevity.

A. Lesion signal intensity remains comparable across the estrus cycle in the NI model. Vaginal cytology was performed on the day of imaging in order to determine if estrus stage has any impact on lesion growth / bioluminescent signal intensity.

B. Impact on estrus stage of donor and recipient mice on lesion longevity. Vaginal cytology was performed on donor mice the day uterine material was collected for transfer to recipient mice. Vaginal cytology was also performed on recipient mice on the day of endometrial tissue receipt. 100% of mice that received estrus stage endometrium still had lesions on d21 post tissue injection.

Fig. S2. Ovarian weight is significantly decreased in mice treated with Cetrorelix.

A. Ovary weights are significantly decreased in Cetrorelix treated mice with induced endometriosis (vehicle n=10, Cetrorelix n=100. Statistical testing was performed using a one-way ANOVA and Tukey’s multiple comparison test. **:p<0.01.